ORIGIN AND DEVELOPMENT OF FLORAL BUDS IN TOBACCO EXPLANTS

Organization of microtubules and microfibrils in differentiating cells

Frans Wilms

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Organization of microtubules and microfibrils

in differentiating cells

een wetenschappelijke proeve op het gebied van de

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CONTENTS:

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I. INTRODUCTION

CHAPTER 1.

INTRODUCTION

Organogenesis is defined as the appearance of a new organ initially a protrusion at a site where only a parent structure was present (Green 1980) It depends on both internal factors, like hormone concentrations, endogenous rhythms and cell–cell interrelationships, and environmental conditions, like light–dark regime, humidity and temperature Since internal factors cannot be controlled *in vivo,* organogenesis and differentiation are preferably studied in *in vitro* systems (reviews McDaniel 1984, Chandler & Thorpe 1986, Williams & Maheswaran 1986) Several *in vitro* systems like tissue explants, callus cultures and cell suspensions have been used and aspects of organogenesis like cell division (Venverloo *et al* 1980) cell differentiation (Hardham 1982) xylogenesis (Falconer *et al* 1985) root, shoot and flower formation (Lyndon & Cunninghame 1986) can be studied

The formation of new organs requires changes in cell polarity and formation of new polarity axes Factors accompanying cell polarity and cell shape are organization of cortical microtubules and cellulose microfibrils (Green 1980, Gunning & Hardham 1982, Hardham 1982) Cell division and place and plane of the future cell walls in plant cells depend on the organization of cortical microtubules and preprophase bands (reviews Gunning & Hardham 1982, Dustin 1984) whereas cell expansion in plant cells depends on cellulose reinforcing patterns in cell walls (reviews Green 1980, Taiz 1984)

Formation of new polarity axes in tissues may be induced by signals established between two regions of a tissue (Williams & Maheswaran 1986) These locally occurring signals, like hormone differences (Williams & Maheswaran 1986 Green 1988) or ionic corrents and Ca^{2+} gradients (Weisenseel & Kicherer 1981) may result in ordered microtubules and microfibrils and eventually in ordered cell division and elongation (Quader *et al* 1986)

In many cells, microtubules have been found parallel to the nascent microfibrils and they have therefore been hypothesized to orient microfibrils during their deposition (reviews Robinson & Quader 1982 Lloyd 1984, Hepler 1985) Recent articles on this subject include Mueller & Brown 1982 Seagull 1983, Hogetsu 1986 Sassen & Wolters–Arts 1986 Bergfeld 1988, Giddings & Staehelin 1988 However, recent observations on algae and higher plants indicate that there is no control of microtubules on microfibrils deposition (Emons 1982 Hahne & Hoffman 1985 Derksen 1986 Okuda & Mizuta 1987 Hayano *et al* 1988 Preston 1988 Traas & Derksen 1989)

Aim of this thesis

This thesis concerns studies on morphogenesis and organogenesis of floral buds in tissue explants of *Nicotiana tabacum* Attention is focussed on the organization of the microtubular cytoskeleton and the cellulose microfibril deposition since they are involved in cell polarity (see above) A mutual relationship between cellulose microfibrils and microtubules is examined

Organogenesis in tobacco explants (Tran Thanh Van 1973), requires two different hormones, an auxin and a cytokinin at particular concentrations (Van den Ende *et al* 1984a Smulders *et al* 1988, Van der Krieken *et al* 1988) and, moreover, depends on age of the parent tissue (Van den Ende *et al* 1984a, Croes *et al* 1985) and the size of the explants (Tran Thanh Van 1977) Under appropriate conditions, floral buds will develop at predictable sites on the

explants (Van den Ende *et al* 1984b)

Initiation and formation of floral buds on tissue explants of *Nicotiana tabacum* is described in relation to the size of the explants the hormone concentrations in the medium and the viscosity of the medium and the sensitivity of the explants to hormones (Chapter 2 and 3) In chapter 3, wound response at the cut surfaces of the explants is examined in relation to the viscosity of the medium

The relationship between organization of cortical microtubules and cell polarity was studied by means of indirect immunofluorescence (Chapter 4 and 5) In chapter 4, the first 2 days of tissue culture is described De-differentiation and the first signs of re-differentiation in the cortex cells of tobacco explants is discussed In Chapter 5 a description of the first events in floral primordia formation is given The establishment of new polarity axes in relation to orientation of cortical microtubules is emphasized

Using replica techniques, the organization of cellulose microfibrils is examined before and after microtubule-depolymerizing drug treatment (cremart and colchicine) and ethylene treatment The relationship between microtubules and microfibrils in cell differentiation is discussed (Chapter 6)

Chapter 7 describes a method for visualization of cytoplasmic elements and cellulose microfibrils in the same preparation Polyethylene glycol embedded material was sectioned conventionally stained for electron microscopy critical point dried and cleaved

Finally chapter 8 summarizes the conclusions of this thesis

References

Bergfield R, Speth V, Schopfer P (1988) Reorientation of microfibrils and microtubules at the outer epidermal wall of maize coleoptiles during auxin-mediated growth Botanica Acta 101 57-67

Chandler SF, Thorpe TA (1986) Hormonal regulation of organogenesis *in vivo* In Purohit SS (ed) Hormonal regulation of plant growth and development Volume 3, pp 1-28 Agro Botanical Publishers (India)

Croes AF Creemers-Molenaar T Van den Ende G, Kemp A Barendse GWM (1985) Tissue age as an endogenous factor controlling *in vitro* bud formation on explants from the inflorescence of *Nicotiana tabacum* L J Exp Bot 36 1771-1779

Derksen J (1986) Cytoskeletal control of cellulose microfibril deposition In Vian B, Reiss D Goldberg R (Eds) Cell Walls 1986 Proceedings of the 4th Cell Wall meeting, Paris 1986 Groupe Paris, France

Emons AMC (1982) Microtubules do not control microfibril orientation in helicoidal cell wall Protoplasma 113 85-87

Falconer MM Seagull RW (1985) Immunofluorescent and calcofluor white staining of developing tracheary elements in *Zinnia elegans* L suspension cultures Protoplasma 125 190-198

Giddings TH jr, Steahelin LA (1988) Spatial relationship between microtubules and plasma-membrane rosettes during the deposition of primary wall microtubules in *Closterium* sp Planta 173 22-30

Green PB (1980) Organogenesis A biophysical view Ann Rev Plant Physiol 31 51-82

Green PB (1988) A theory for inflorescence development and flower formation based on morphlogical and biophysical analysis in *Echeveria* Planta 175 153-169

Gunning BES, Hardham AR (1982) Microtubules Ann Rev Plant Physiol 33, 651-698

Hahne G Hoffmann F (1985) Cortical microtubular lattices Absent from mature mesophyll and necessary for cell division? Planta 166 309-311

Hardham AR (1982) Regulation of polarity in tissues and organs In Lloyd CW (ed) The Cytoskeleton in Plant Growth and Development pp 377-403 Academic Press, London

Hayano S, Itoh T, Brown RM jr (1988) Orientaion of microtubules during regeneration of cell wall in selected giant marine algae Plant Cell Physiol 29 785-793

Hepler PK (1985) The plant cytoskeleton In Robards AW (ed) Botanical Microscopy 1985 pp 233-262 Oxford Univ Press

Hogetsu T (1986) Orientation of wall microfibril deposition in root cells of *Pisum sativum* L var Alaska Plant Cell Physiol 27 947-951

Lloyd CW (ed) (1982) The cytoskeleton in plant

growth and development Academic Press, London

Lloyd CW (1984) Towards a dynamic helical model for the influence of microtubules on wall patterns in plants Int Rev Cytol 86 1–35

Lyndon RF, Cunninghame ME (1986) Control of shoot apical development via cell division Symposium Society for Experimental Biology 40 233–255

McDaniel CN (1984) Competence, determination and induction in plant development In Malacinski GM (Ed) Pattern formation A Primer in Developmental Biology pp 393–412 Macmillan Co , New York

Mueller SC Brown RM jr (1982) The control of cellulose microfibril deposition in the cell wall of higher plants I Can directed membrane flow orient cellulose microfibrils? Indirect evidence from freeze–fractured plasma membranes of maize and pine seedlings Planta 154 489–500

Okuda K Mizuta S (1987) Modification in cell shape unrelated to cellulose microfibril orientation in growing thallus cells of *Cheatomorpha moniligera* Plant Cell Physiol 28 461–473

Preston RD (1988) Cellulose–microfibril–orienting mechnisms in plant cell walls Planta 174 67–74

Quader H Deichgraber G, Schnepf E (1986) The cytoskeleton of *Cobaea* seed hairs Patterning during cell–wall differentiation Planta 168 1–10

Robinson DG Quader H (1982) The microtubule–microfibril syndrome In Lloyd CW (ed) The Cytoskeleton in Plant Growth and Development pp 109–126 Academic Press, London

Sachs, T (1981) Polarity changes and tissue organization in plants In Schweiger, H G (ed) International Cell Biology 1980–1981 pp 489–496 Springer Verlag, Berlin Heidelberg New York

Sassen MMA, Wolters–Arts AMC (1986) Cell wall texture and cortical microtubules in growing staminal hairs of *Tradescantia virginiana* Acta Bot Neerl 35 351–360

Seagull RW (1983) The role of the cytoskeleton during oriented microfibril deposition I Elucidation of the possible interaction between microtubules and cellulose synthetic complexes J Ultrastruct Res 83 168–175

Smulders MJM Janssen GFE Croes AF, Barendse GWM, Wullems GJ (1988) Auxin regulation of flower bud formation in tobacco explants J Exp Bot 39 451–459

Taiz L (1984) Plant cell expansion Regulation of cell wall mechanical properties Ann Rev Plant Physiol 35 585–657

Traas JA, Derksen J (1989) Microtubules and cellulose microfibrils in plant cells simultaneous demonstration in dry cleave preparations Eur J cell Biol 48 159–164

Tran Thanh Van K (1973) Direct flower bud neoformation from superficial tissue of small explants of *Nicotiana tabacum* L Planta 115 87–92

Tran Thanh Van K (1977) Regulation of morphogenesis In Barz W, Reinhard E, Zenk MH (eds) Plant tissue culture and its bio–technological application pp 367–385 Springer, Berlin Heidelberg New York,

Van den Ende G Croes AF, Kemp A, Barendse GWM (1984a) Development of flower buds in thin–layer cultures of floral stalk tissue from tobacco role of hormones in different stages Physiol Plant 61 114–118

Van den Ende G, Croes AF, Kemp A Barendse GWM Kroh M (1984b) Development of flower buds in thin–layer tissue cultures of *Nicotiana tabacum* Physiol Plant 62 83–88

Van der Krieken WM Croes AF, Barendse GWM Wullems GJ (1988) Uptake and metabolism of benzyladenine in early stage of flower bud development *in vitro* in tobacco Physiol Plant 74 113–118

Venverloo CJ, Hovenkamp PH, Weeda AJ Libbenga KR (1980) Cell division in *Nautilocalyx* explants I Phragmosome, preprophase band and plane of cell division Z Pflanzenphysiol 100 161–174

Weisenseel MH, Kicherer RM (1981) Ionic currents as control mechanism in cytomorphogenesis In Kiermayer O (ed) Cytomorphogenesis in plants pp 379–399 Springer–Verlag Wien New York

Williams EG, Maheswaran G (1986) Somatic embryogenesis Factors influencing coordinated behaviour of cells as an embryogenic group Ann Bot 57 443–462

II. ORIGIN AND DEVELOPMENT OF FLORAL BUDS AND DEPOSITION OF SUBERIN IN TOBACCO EXPLANTS

CHAPTER 2.

ORIGIN AND DEVELOPMENT OF FLORAL BUDS IN TOBACCO EXPLANTS

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ORIGIN AND DEVELOPMENT OF FLORAL BUDS IN TOBACCO EXPLANTS

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SUMMARY

Initiation and formation of floral buds was studied on explants of *Nicotiana tabacum*. During the first stage of development (0 to 4 d), protrusions are formed at the basal side of the explant as a result of cell divisions in both sub-epidermal and epidermal cell layers. The second stage (4 to 7 d) is characterized by the formation of tracheary centres inside the protrusions followed by the formation of floral primordia at the surface of the protrusions. These primordia result from both epidermal and sub-epidermal cell divisions. In the final stage (7 to 15 d), flower primordia develop into flower buds and tracheary elements grow into the buds. Polarity present in cells of freshly cut explants is lost within a few days after the onset of the experiment. After 4 to 7 d, a new polarity axis has differentiated inside the protrusion. This axis runs from the tracheary structure inside the protrusion to the flower primordium at the periphery.

Key words: Tobacco, bud formation, polarity, explants, primordia

INTRODUCTION

Differentiation in plants depends on both environmental conditions, such as light–dark regime, humidity and temperature, and internal factors such as hormone concentrations, endogenous rhythms and cell–cell interrelationships. Since internal factors cannot be controlled *in vivo*, embryogenesis and differentiation often have been studied in *in vitro* systems (for reviews see Tran Thanh Van, 1977 and Williams & Maheswaran, 1986).

As shown by Tran Thanh Van (1973), tissue explants of *Nicotiana tabacum* can directly develop various organs *in vitro*. Even the development of highly specialized organs like inflorescences can be studied.

The formation of floral buds on *N. tabacum* explants as described by Nguyen Thi Dien & Tran Thanh Van (1974) occurs by numerous cell divisions in the sub-epidermal layer, resulting in a meristematic 'dome' or 'knob' on which the actual floral buds develop, followed by a differentiation of tracheids in the explanted tissue and in the newly formed meristematic knob.

It has been shown by van den Ende *et al.* (1984c) that the site of floral bud formation depends on the hormone concentrations in the medium. Also, the origin of the meristematic knob, sub-epidermal and epidermal *vs* only sub-epidermal, appeared to depend on the hormone concentrations in the medium (van der Ende *et al.*, 1984b).

Here we describe in some detail the formation of floral buds under conditions as described by van den Ende *et al.* (1984a). We have studied the influence of the size of the explants on the frequency of response and the site of bud formation,

This paper is dedicated to Prof. Dr H. F. Linskens (Nijmegen) on the occasion of his 65th birthday in 1986.

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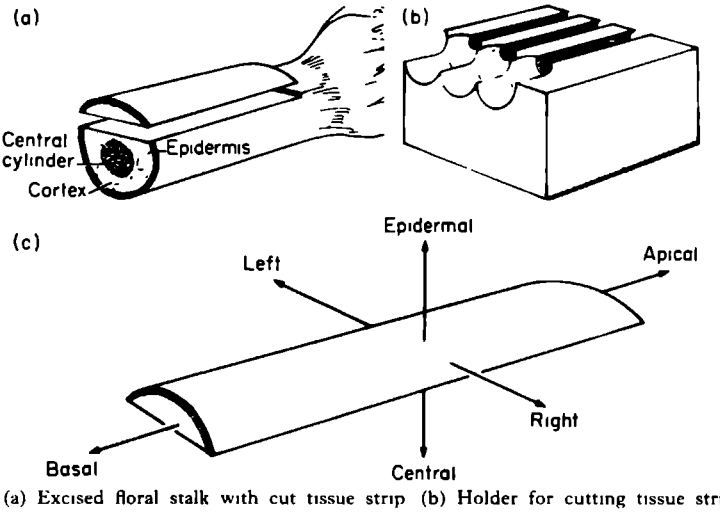


Fig 1 (a) Excised floral stalk with cut tissue strip (b) Holder for cutting tissue strips. (c) Topography of cut tissue strip The strip has the same orientation as in Figure 1(a).

the development of new polarity axes in the meristematic region (= protrusion), and we traced the origin of the protrusions. The results are discussed in relation to bud formation.

MATERIALS AND METHODS

Preparation and culture of tissue explants

Flower stalks attached to flowers at anthesis were collected from inflorescences of *Nicotiana tabacum* (van den Ende *et al.*, 1984a). Tissue strips [Fig.1(a)] were cut from sterilized stalks using a holder [Fig.1(b)] with various grooves to accommodate differences in diameter of the floral stalks. The size of the explants was 0·48 to 0·96 mm by 10 mm, and the thickness varied from four to eight cell layers. Explants were transplanted on a Murashige and Skoog medium (Murashige & Skoog, 1962) in agar, supplied with 10^{-7} M α-naphthalene acetic acid (NAA), 10^{-6} M benzyl-amino-purine (BAP) and 150 mM glucose. Explants cultured on 10^{-7}M NAA, 10^{-7} M BAP and 150 mM glucose did not develop flower buds and were used as a control (van den Ende *et al.*, 1984b). We studied only the basal side, proximal to the main axis of the explants as only there will buds develop under the present conditions (van den Ende *et al.*, 1984c).

Examination of whole explant

The terminology used to describe the various sides of the tissue strips is indicated in Figure 1(c).

Differential-interference contrast microscopy

Explants were collected at various intervals, treated with 4% NaOH for 6 h at 55 °C and further treated in clearing fluid according to the method of Herr (1971). After 24 h at 55 °C, the explants were placed in glycerol for storage. The xplants were transported to welled glass slides and embedded in glycerol for xamination. Optical sections were made using a differential-interference contrast ıicroscope.

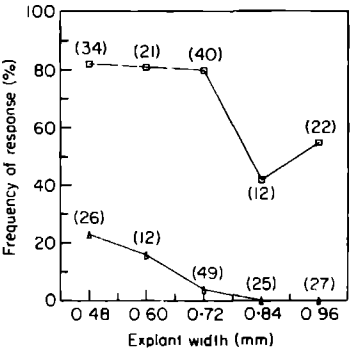


Fig 2 Frequency of bud-forming response related to explant width Numbers in parentheses, number of explants used □, 10^{-7} M NAA/10^{-6} M BAP, △, 10^{-7} M NAA/10^{-7} M BAP

Scanning electron microscopy

Explants were fixed with 3 % glutaraldehyde in 0·1 M Na-Cacodylate buffer (pH 7·2) at room temperature. After 2 h they were washed in water, dehydrated slowly in ethanol, critical point-dried (Balzers Union, Mq 202) and sputtered with gold using a Balzers Union sputter apparatus (type Tpg 031) The explants were examined with a scanning electron microscope (JSM–U3 or JSM–T300).

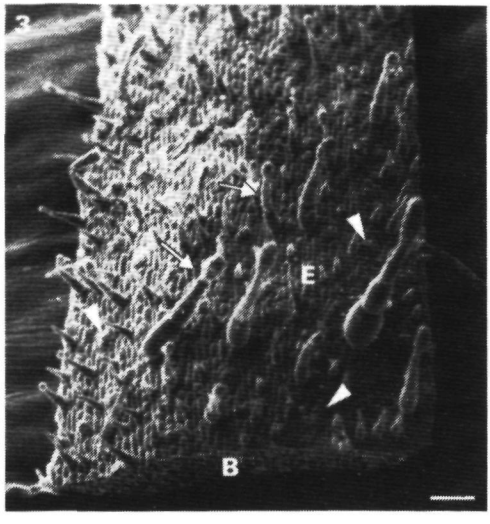
Examination of sections

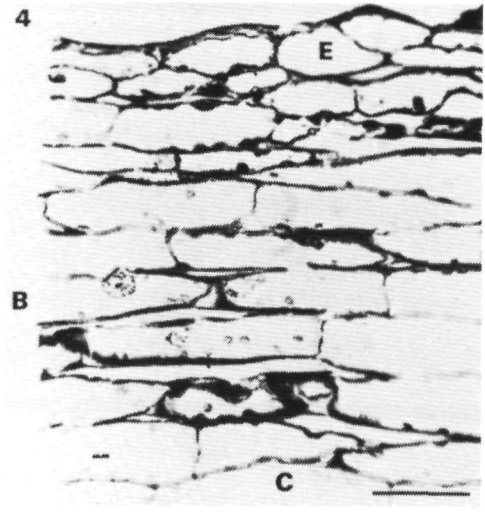
Explants were pre-fixed in 3 % glutaraldehyde in 0·1 M Na-Cacodylate buffer (pH 7 2) for 2 h and fixed in 2 % OsO_4 and 3 % glutaraldehyde in 0·1 M Na-Cacodylate buffer (pH 7 2) for 2 h Both fixations were at room temperature. The explants were washed in water, dehydrated slowly in ethanol and embedded in Spurr's resin (Spurr, 1969). For light microscopy, 1 μm serial sections (both longitudinal and cross-sections) were made with glass knives on a Sorval MT 5000 or a Sorval Porter-Blum ultramicrotome (type NT–2). Sections were stained with toluidine blue for 1 min and enclosed in Euparal. The slides were examined under a Leitz microscope. A three-dimensional reconstruction of an explant was made from cross-sections using a KONTRON MOP-Videoplan. Input of the contours of the different cell types of the serial sections was done using a HIPAD X–Y tablet.

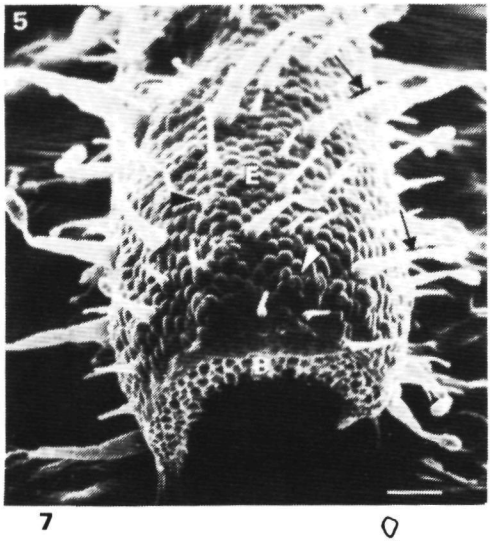
Results

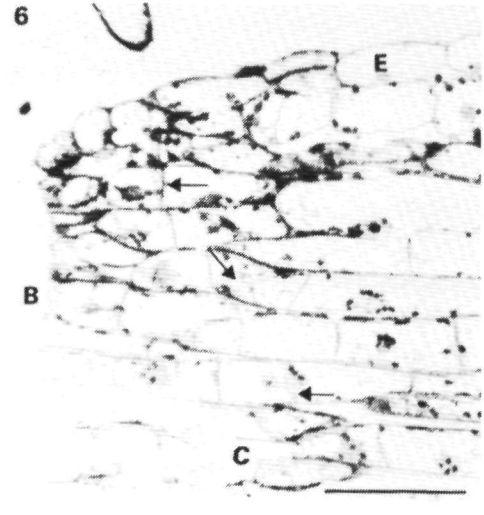
Effect of size of explants on bud formation

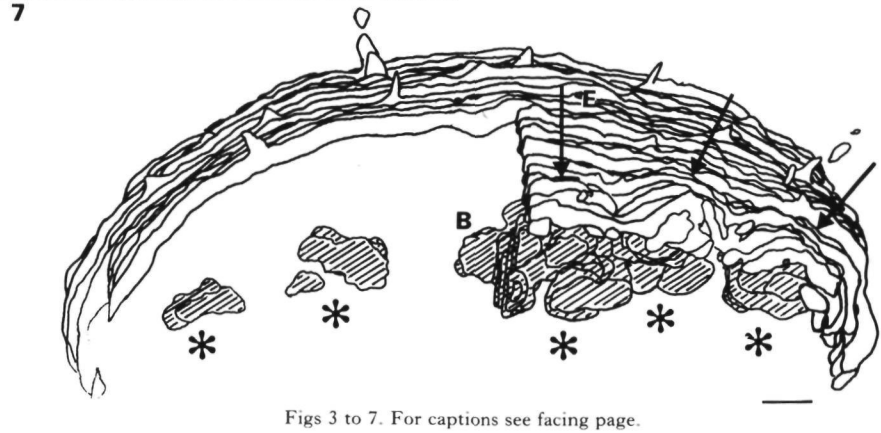
The influence of the size of the explants on floral bud formation was determined by measuring the frequency of response of bud-forming explants in relation to the width (from the left to the right side) of the explants. The number of bud-forming explants is highest in explants less than 0·72 mm wide (Fig 2). The number of buds per explant on smaller explants is not significantly lower than on wider explants. The mean number of flower buds per explant is about 4·8. With a few exceptions, control explants incubated at the low BAP concentration develop no flower buds. Only thin explants (not wider than 0 72 mm) occasionally form floral buds under these conditions (Fig 2). However, never more than two buds are formed per explant. For further studies, we used explants of about 0·70 mm in width.











Figs 3 to 7. For captions see facing page.

Development of the floral buds on the explant

The surface of a freshly cut explant is characterized by regularly shaped epidermal cells (Fig. 3) Besides the epidermis, the explant consists of several layers of cortical cells; no tracheary elements are present (Fig 4).

Two days after the onset of the experiment, the explant is curved due to a difference in expansion of the epidermal cells, the underlying cortical cells and the cut surface at the central side The originally ribbed epidermal surface has almost disappeared and the cells have become spherical (Fig 5) At all the cut surfaces, cell divisions occur that will result in the formation of callus after about 4 d (Fig. 6) Both epidermal and cortical cells divide At the basal side, the plane of the new cell walls is mostly perpendicular or oblique to the long axis of the explant. At the apical side of the explant, fewer cell divisions occur compared to the basal side.

After 4 d, cells at the basal side of the tissue have lost their original basal–apical polarity and their differentiated character. The first cell divisions are almost immediately followed by further, mostly oblique, divisions one to two cells deeper in the explant at the entire basal surface (Fig 8) Further cell divisions occur throughout this zone, up to 0 3 mm from the basal side, their direction shifting from oblique to irregular Sections show distinct meristematic centres at the surface and inside the explant forming the protrusions. The spatial position of these centres in the basal zone was determined by a 3-D reconstruction of cross-sections (Fig. 7). Only one single row of five protrusions is formed (three protrusions are shown) Meristematic centres inside the explant are always spatially related to the protrusions visible at the surface of the explant. During the following 2 d, tracheary elements are formed inside the protrusions which develop into tracheary centres.

After 7 d the protrusions have increased in size and on the surface floral primordia become visible (Fig 9). The primordia result from groups of cells of both epidermal and cortical origin (Fig. 10). As in the formation of the protrusions, here, too, epidermal cells partake in the development of the buds by dividing parallel to the surface of the tissue. The dividing cells are very small and rich in cytoplasm, and they also show mitotic figures. Inside the protrusion, tracheary centres have increased in size (Fig. 11). Floral primordia appear after the formation of tracheary centres, displaying a new polarity axis (epidermal–central) within the protrusion (Fig 10). On one protrusion, up to two primordia can be formed

Within the following 5 d, the floral primordium differentiates into a bract and an acropetally developing flower Underneath the bud, the well-developed tracheary system is visible (Fig 12) This system extends into the different parts of the flower bud by differentiation of cells into tracheary elements Tracheary

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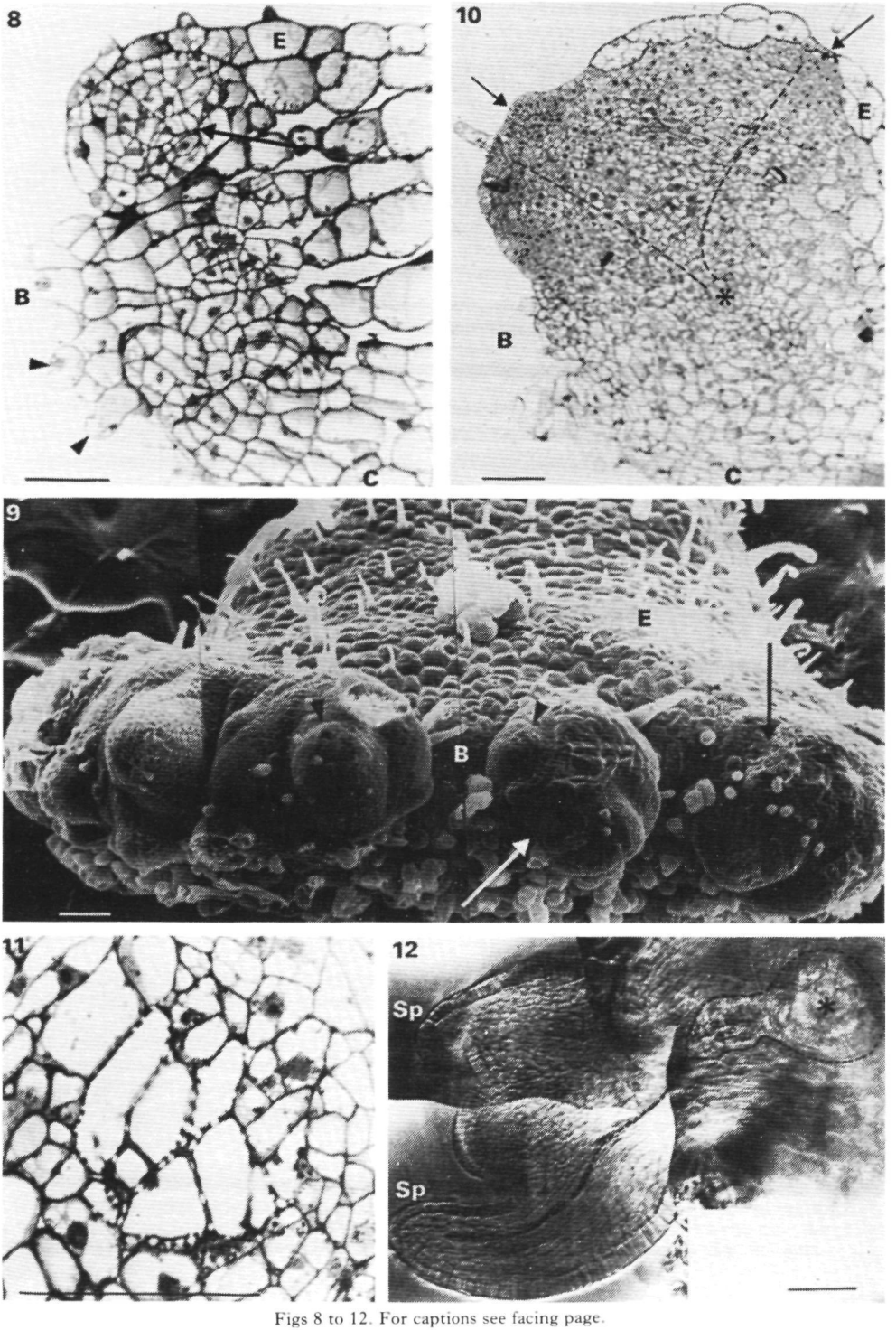
Fig 3 Scanning electron micrograph of freshly cut explant Arrows indicate glandular hairs, arrowheads indicate stomata B, basal side, E, epidermal side, bar = 0 1 mm

Fig 4 Light micrograph of a longitudinal section of a freshly cut explant B, basal side, C, central side, E epidermal side, bar = 0 1 mm

Fig 5 Scanning electron micrograph of a 2 d old explant Arrows indicate glandular hairs, arrowheads indicate stomata B, basal side, bar = 0 1 mm

Fig 6 Light micrograph of a longitudinal section of a 2 d old explant Arrows indicate new cell walls B, basal side, C, central side, E, epidermal side, bar = 0 1 mm

Fig 7 3-D reconstruction of a 4 d old explant The central side is not shown Distance between each section 0 02 mm Arrows indicate protrusions, *, meristematic centre B, basal side, E, epidermal side, bar = 0 1 mm



Figs 8 to 12. For captions see facing page.

centres may become connected to each other, forming a tracheary bundle parallel to the basal side of the explant.

The development of explants on control medium is at first the same as on experimental medium: the epidermal cells become spherical after 1 d, and cell divisions occur at the cut surfaces. However, only little callus and no protrusions are formed. Only incidentally one or two protrusions appear on small explants (about 0·7 mm wide). Exceptionally, after 15 d of culturing a flower may be formed on a protrusion. Whenever protrusions appear, tracheary elements are also visible inside the protrusions.

Discussion

The efficiency of *in vitro* flower bud formation depends on both hormone concentration of the medium (Venverloo, 1976; Hillson & Lamotte, 1977; Tran Thanh Van, 1977; van den Ende *et al.*, 1984b) and the size of the explants (Tran Thanh Van, 1977). The polar bud formation, however, is not directly related to the size of the explants: they develop invariably at the basipetal side of the explants.

The first cell divisions occurring at the cut sides are always parallel to the wounded surfaces, as has also been observed in *Nautilocalyx lynchii* explants (Venverloo & Pronk, 1982). Wounding probably is the initiating factor for cell division (Venverloo & Pronk, 1982) and de-differentiation (Lipetz, 1970; Williams & Maheswaran, 1986). Evidence exists that somatic embryogenesis *in vitro* is caused by a de-repression of embryogenic potential after wounding (Williams & Maheswaran, 1986). Correspondingly, the effect of wall oligosaccharins on regeneration of tobacco explants, as described by Albersheim & Darvill (1985), may be part of a physiological response to wounding resulting in a de-repression of cell potential. Wound reactions may also account for the effect of explant size on bud formation, the wound surface being relatively large in smaller explants. In this context, we consider the unexpected formation of flower buds on small control explants especially important. The polar distribution of flower buds may depend on a stronger wound reaction at the basal side, since most cell divisions occur there. The basipetal NAA transport may be another, very important possible reason for the polar distribution (van den Ende *et al.*, 1984c; Smulders, pers. comm.).

In our system, flower bud formation is a two-step process. First, protrusions are formed with tracheary centres. After that, flower buds protrude from meristematic regions at the surface of the protrusions. Not only sub-epidermal cells partake in the formation of buds, but also epidermal cells and several layers of cortical cells. Our findings are in contrast with the results of Nguyen Thi Dien & Tran Thanh Van (1974) and van den Ende *et al.* (1984c). Nguyen Thi Dien & Tran Thanh Van describe protrusions as originating from the cortical layer just

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Fig. 8 Light micrograph of a longitudinal section of a 4 d old explant Arrow indicates protrusion; arrowheads indicate callus cells B, basal side, C, central side, E, epidermal side, bar = 0·1 mm.

Fig 9. Scanning electron micrograph of a 7 d old explant. Arrows indicate protrusions; arrowheads indicate flower primordia B, basal side, E, epidermal side, bar = 0 1 mm

Fig 10 Light micrograph of a longitudinal section of a 7 d old explant Arrows indicate flower primordia; *, tracheary centre, dotted line, polarity axis, B, basal side, C, central side, E, epidermal side; bar = 0·1 mm

Fig 11 Light micrograph of a longitudinal section of a tracheary centre of a 7 d old explant Bar = 0·1 mm

Fig 12 Light micrograph of an optical section of a 15 d old explant Dotted line, tracheary elements, *, tracheary centre; SP, sepal, bar = 0·1 mm.

beneath the epidermis. Van den Ende *et al.* described two types of bud formation. (1) From epidermis and several underlying cells, forming a protrusion. Flower buds are initiated sub-epidermally. (2) From sub-epidermal layers, forming a protrusion. Flower buds are formed sub-epidermally and the epidermal cells only elongate. It is, however, hard to believe that the epidermis does not partake in flower bud formation. The epidermis is probably important for re-differentiation, since its presence is required in order to establish a tunica-corpus structure.

The location of the first developing tracheary system in our material is clearly related to the site of protrusion formation. Tracheary centres are formed exclusively basally, eventually connecting with each other, forming strands perpendicular to the long axis of the explant. Tracheary centres are necessary for the formation of flower buds, No buds are formed on protrusions without tracheary centres. In the material described by Nguyen Thi Dien & Tran Thanh Van (1974), meristematic knobs (= protrusions) are found all over the edges of the tissue, and correspondingly axial tracheary elements are found. We never observed an effect of the presence of hairs and stomata on the site of cell divisions as shown for *N. lynchii* (Tran Thanh Van & Drira, 1971; Venverloo, 1976; Venverloo, Koster & Libbenga, 1983). Owing to their abundance on the original surface, they are expected to be present on the surface of the protrusions.

With the present technique, we cannot trace the origin of protrusions and buds any further. These structures probably result from groups of meristematic cells, the sites of which may depend on cell–cell interactions. Such interactions, for example lateral inhibition, lead in general to pattern formation (Williams & Maheswaran, 1986).

With the onset of the development of buds and the formation of xylem elements, new polarity axes are established in the protrusions. At this stage, the situation roughly resembles the *in vivo* bud formation: establishment of an apical meristematic dome, subsequently followed by delineation of a procambium strand and xylem and finally the appearance of acropetally developing flowers (Hicks & Sussex, 1970).

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REFERENCES

ALBERSHEIM, P & DARVILL, A G (1985) Oligosaccharins *Scientific American*, **253**, 44–50

ENDE, G. VAN DEN, BARENDSE, G. W M , KEMP, A. & CROES A F (1984a) The role of glucose on flower bud formation in thin-layer tissue cultures of *Nicotiana tabacum* L. *Journal of Experimental Botany*, **35**, 1853–1859

ENDE, G VAN DEN, CROES, A F , KEMP, A & BARENDSE, G W M (1984b) Development of flower buds in thin-layer cultures of floral stalk tissue from tobacco role of hormones in different stages. *Physiologia Plantarum*, **61**, 114-118.

ENDE, G VAN DEN, CROES, A F , KEMP, A , BARENDSE, G W. M & KROH, M (1984c) Floral morphogenesis in thin-layer tissue cultures of *Nicotiana tabacum Physiologia Plantarum*, **62**, 83 88

HERR, J M. (1971) A new clearing-squash technique for the study of ovule development in angiosperms *American Journal of Botany*, **58**, 785 790

HICKS, G S & SUSSEX, I M (1970) Development in vitro of excised flower primordia of *Nicotiana tabacum Canadian Journal of Botany*, **48**, 133 139

HILLSON, T P & LAMOTTE, C E (1977) In vitro formation and development of floral buds on tobacco stem explants Effects of kinetin and other factors *Plant Physiology*, **60**, 881 884

LIPETZ, J (1970) Wound-healing in higher plants *International Review of Cytology*, **27**, 1 28

MURASHIGE, T & SKOOG, F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture *Physiologia Plantarum*, **15**, 373 397

NGUYEN THI DIEN & TRAN THANH VAN, M (1974) Differenciation in vitro et de novo d'organes floraux directement a partir des couches minces de cellules de type epidermique de *Nicotiana tabacum* Etude au niveau cellulaire *Canadian Journal of Botany*, **52**, 2319–2322

SPURR, A R (1969) A low-viscosity epoxy resin embedding medium for electron microscopy *Journal of Ultrastructural Research*, **26**, 31–43

TRAN THANH VAN, K (1977) Regulation of morphogenesis In *Plant Tissue Culture and its Bio-Technological Applications* (Ed by W Barz, E Reinhard & M H Zenk), pp 367–385 Springer-Verlag, Berlin, Heidelberg

TRAN THANH VAN, M (1973) Direct flower neoformation from superficial tissue of small explants of *Nicotiana tabacum* L *Planta*, **115**, 87–92

TRAN THANH VAN, M & DRIRA, A (1971) Definition of a simple experimental system of directed organogenesis de novo organ neoformation from epidermal tissue of *Nautilocalyx lynchei* In *Les Cultures de Tissus de Plantes*, Coll Intern C N R S 193 Strasbourg, 1970, C N R S , Paris, 1971

VENVERLOO, C J (1976) The formation of adventitious organs III A comparison of root and shoot formation on *Nautilocalyx* explants *Zeitschrift fur Pflanzenphysiologie*, **80**, 310-322

VENVERLOO, C J , KOSTER, J & LIBBENGA, K R (1983) The formation of adventitious organs IV The ontogeny of shoots and leaves from epidermis cells of *Nautilocalyx lunchii Zeitschrift fur Pflanzen-physiologie*, **109**, 55–67

VENVERLOO, C J & PRONK, N (1982) Regulation of the plane of division in epidermis cells of *Nautilocalyx* leaf explants In *Plant Tissue Culture 1982* (Ed by Akio Fujiwara), pp 49–50 Proceedings of the Fifth International Congress for Plant Tissue & Cell Culture

WILLIAMS, E G & MAHESWARAN, G (1986) Somatic embryogenesis factors influencing co-ordinated behaviour of cells as an embryogenic group *Annals of Botany*, **57**, 443 462

CHAPTER 3

SUBERIZATION AND BUD FORMATION IN TOBACCO EXPLANTS.

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SUMMARY

Wound-healing and floral bud formation were studied in explants of *Nicotiana tabacum*. Examination of sectioned material by means of conventional light microscopy, fluorescence microscopy and electron microscopy showed that suberin is formed in cells at the cut surface most close to the medium after 4-7 days of culture.

The number of cells with suberin depositions was dependent on the agar concentration in the medium. In explants cultured on medium with low agar concentration (0.2% agar) less cells with suberin deposition and more callus cells are found without suberin. Explants cultured on medium with high agar concentration (5% agar) formed a complete layer of cells with suberin deposition.

In tobacco explants floral determination occurred within 4 days after explantation. It is concluded that the incapacity of the explants to form buds is probably due to barrier formation by suberin.

INTRODUCTION

Wounding and wound-healing is a common event in higher plants. The reactions (wound response) of the plants to wounding are diverse but always lead to regeneration of a part or all of the functions which were previously shared by the damaged or lost cells, tissues or organs (review Lipetz 1970). Regeneration implies initiation of cell divisions, formation of protective layers and regeneration of vascular elements (for reviews see Biggs 1984, Imaseki 1985).

One of the first visible reactions of tissues and cells to wounding is cell division. The plane of the newly deposited cell walls is parallel to the plane of the wounding (Venverloo *et al.* 1980, Gunning 1982, Wilms & Sassen 1987).

The function of the protective layer is to stop loss of water and to prevent entry of pathogenic organisms at the wounded surfaces (Cline 1983, Kolattukudy 1984). Suberization appears to be the most common response to wounding (Kolattukudy 1981) and depends on temperature, auxin concentrations and humidity (Lipetz 1970, Biggs & Northove 1985).

Suberin can be detected with conventional light microscopy and different staining techniques (Jenssen 1962, Cline & Neely 1983, Eastman *et al.* 1988), fluorescence microscopy (Biggs 1986) and electron microscopy (Ryser *et al.* 1983).

In vitro culture techniques are very suitable to study organogenesis and wound response. Tissue strips of floral stalks of tobacco develop floral buds under appropriate conditions (Wilms & Sassen 1987). Whenever tissue strips are cut, the tissue is wounded and wound responses occur at all cut sides of the explants.

The purpose of this study was to investigate the wound response (i.e. formation of suberin) of cortex cells of tobacco tissue strips at the cut surface. Suberin deposition and floral bud formation in relation to the contact of the explants with the medium were studied. Sensitivity of the explants to induce floral buds was studied by

transferring explants to different media at different times

MATERIALS AND METHODS

Preparation and Culturing

Tissue strips, 0 6 by 7 mm, were cut from floral stalks of *Nicotiana tabacum* as previously described by Wilms & Sassen (1987) and cultured on Murashige and Skoog (M&S) medium (Murashige & Skoog 1962) supplied with 10^{-7}M NAA (1-Naphthalene acetic acid), 10^{-6}M BAP (Benzyl-amino-purine), 125mM glucose and 1% agar On this medium floral buds will develop between 10 and 14 days after explantation As a control tissue strips were cultured on M&S medium supplied with 10^{-7}M NAA 10^{-7}M BAP 125mM glucose and 1% agar On this medium no buds will develop (Wilms & Sassen 1987) For more details concerning origin of the explants polarity and terminology see Wilms & Sassen (1987)

In order to examine a possible influence of the contact of the explants with the medium and the availability of the medium on the wound-healing process and on the floral bud formation the agar concentration in the medium was varied from 0 2% to 5% Medium with 0 2% agar was very fluid and explants were floating on it Medium with 5% agar was very solid and dry and the central parts of the explants had hardly contact with the medium After 14 days the number of buds were counted

To study the sensitivity of the tissue to induce floral buds, strips were cultured for different periods of time on either bud inducing or control medium and then transferred on either control or bud inducing medium respectively After 14 days the number of buds were counted

Light Microscopy

Explants were fixed and embedded in polyethylene glycol 1500 (PEG) as described by Wilms & Derksen (1988) Longitudinal sections (10 μm thick) were made with a steel knife on a Reichert hand microtome and placed on poly-L-lysine coated coverslips The PEG was carefully removed by dipping the coverslips into water

Different dyes for the detection and identification of a protective layer were used Phloroglucinol-HCl for lignin (Gurr 1956, Jensen 1962) Phloroglucinol-HCl and ultraviolet fluorescence microscopy for suberin (Biggs 1985), Sudan III for suberin and cutin (Jensen 1962) and Basic Fuchsin for lignified walls and cutin (Gurr 1956) Autofluorescence of suberized cell walls was examined using ultraviolet light (340-380 nm excitation filter and 430 nm barrier filter) (Biggs 1985) The sections were examined under a Leitz Varia Orthomat combination microscope The absorbtion spectra of different parts of a 7 day old explant were determined by measuring the

Fig 1 Light micrograph of a longitudinal section of a freshly cut explant The epidermis and several cortex cells are visible
Fig 2 Autofluorescent image of a longitudinal section of a 2 d old explant under ultraviolet light First longitudinal cell divisions occur at the central side
Fig 3 Autofluorescent image of a longitudinal section of a 4 d old explant under ultraviolet light Several rows of longitudinally divided cells are visible First signs of autofluorescence in some cells at the central side is found
Fig 4 A,B C) Autofluorescent image of longitudinal section of a 7 d old explant under ultraviolet light
A) General view, showing autofluorescence at the central side of the explant
B) Detailed view of the central side showing cells with no autofluorescence ("callus-cells")
C) Detailed view of the central side showing cells with autofluorescence ("suberin-cells')
D) Light micrograph of a longitudinal (30 m thick) sections of a 7 d old explant after staining with Sudan III "Suberin-cells' are visible
B basal side C central side E epidermal side, arrow suberin containing cell, arrow head callus cell small arrow first signs of longitudinal cell divisions Fig 1-4A, Bar 0 1 mm, Fig 4B-D, 20 μm

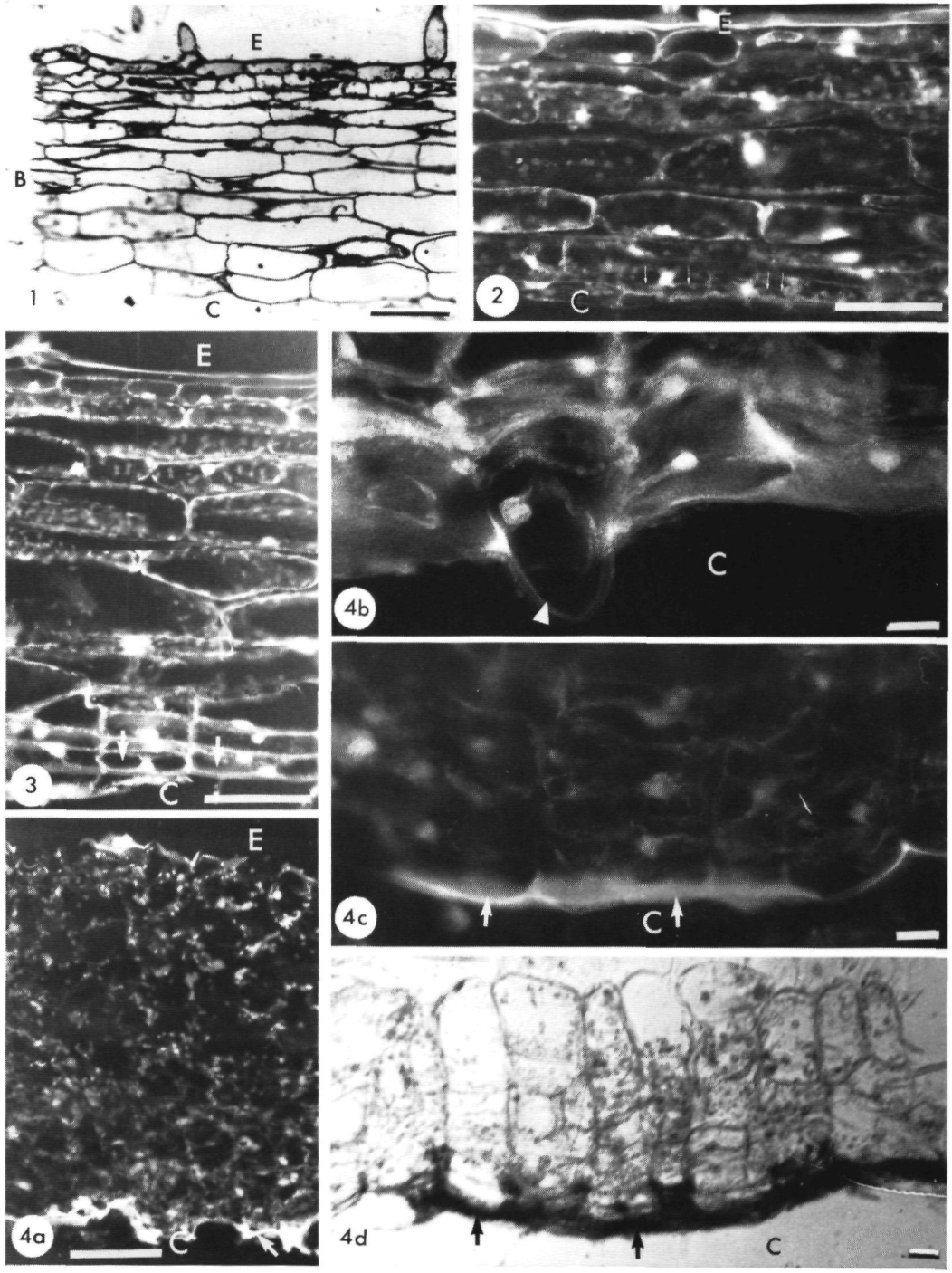
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Table 1 Histochemical colour reactions of cells of 14 days old tobacco explants cultured on medium with 1% agar

Tissue	Phloroglucinol-HCl (light microscopy)	Phloroglucinol-HCl (fluorescence microscopy)	Sudan III	Basic Fuchsine
Cuticle	unstained	fluorescence	red	red
Tracheary elements	red	no fluorescence	unstained	red
Central cells	unstained	fluorescence (some cells no fluorescence)	red	unstained
Expected Colour				
Cutin	unstained	no fluorescence	red	red
Lignin	red	no fluorescence	unstained	red
Suberin	unstained	fluorescence	red	unstained

autofluorescence under ultraviolet light (340-380 nm excitation filter and 430 nm barrier filter) according to Willemse (1981)

Transmission Electron Microscopy

Explants were fixed, dehydrated and embedded in Spurr's resin (Spurr 1969) as described by Wilms & Sassen (1987) Gold coloured longitudinal sections were cut with glass knives on a Sorval MT 5000 and placed on formvar/carbon coated grids The sections were examined in a Philips EM 201 or a JEM 100 CX II electron microscope at 60 kV

RESULTS.

Suberin Formation

Freshly cut explants consist of an epidermis and several layers of cortex cells (Fig 1) No cell divisions were observed After two days of culture on medium containing 1% agar the first cell divisions occurred in cells at the central and basal side of the explants The plane of the new cell walls in cells at the central side was mostly parallel to the longitudinal cell axis (Fig 2) We only focussed on cells at the central and basal

Fig 5 Autofluorescent image of a longitudinal section of a 7 d old explant under ultraviolet light No autofluorescence is visible in cells at the most basal side of the explant

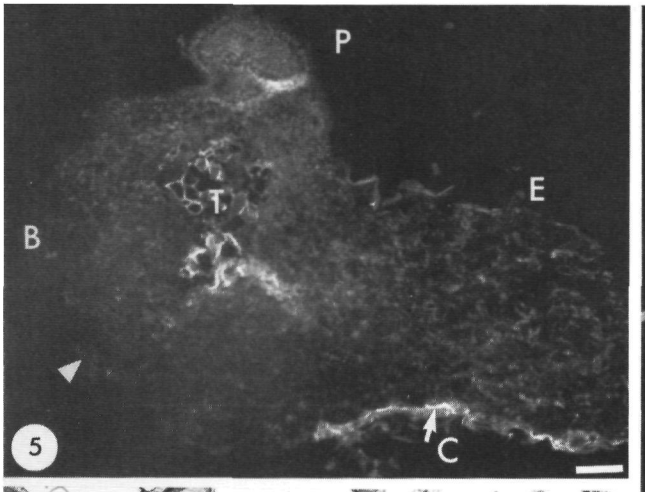
Fig 6 Autofluorescent image of a longitudinal section of a 14 d old explant under ultraviolet light "Suberin-cells" are formed also at the most basal side of the explants

Fig 7 A) General view of an ultrathin longitudinal section of the central side of a 7 d old explant "Suberin-cells" and "callus-cells are visible

B,C) Detailed view of an ultrathin longitudinal section of suberin containing cell at the central side of a 7 d old explant The lamellated region between the cell wall and the plasmamembrane is visible

D) Detailed view of an ultrathin longitudinal section of a "callus-cell" at the central side of a 7 d old explant No lamellated region between the cell wall and the plasmamembrane is visible

B basal side, C central side, Cy cytoplasm E epidermal side F floral bud, P floral primordium S suberin layer, T tracheary centre V vacuole W cell wall arrow suberin containing cell arrow head callus cell Fig 5 6, Bar 0 1 mm, Fig 7A, Bar 5 μm, Fig 7B,D, Bar 1 μm, Fig 7C, Bar 0 1 μm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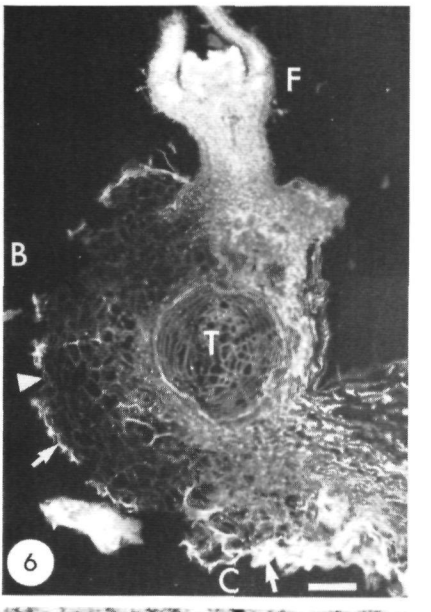
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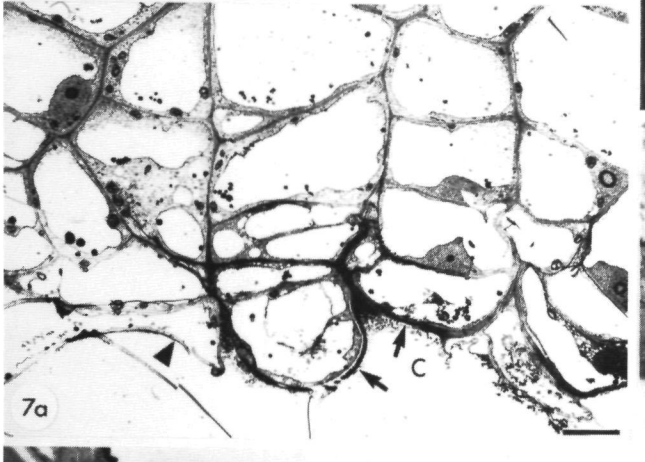
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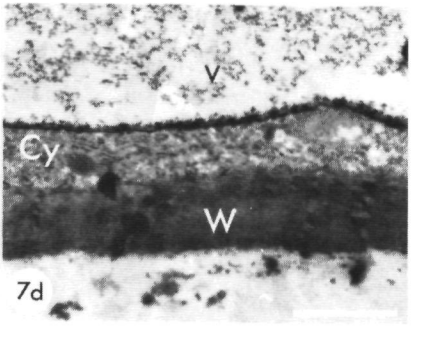
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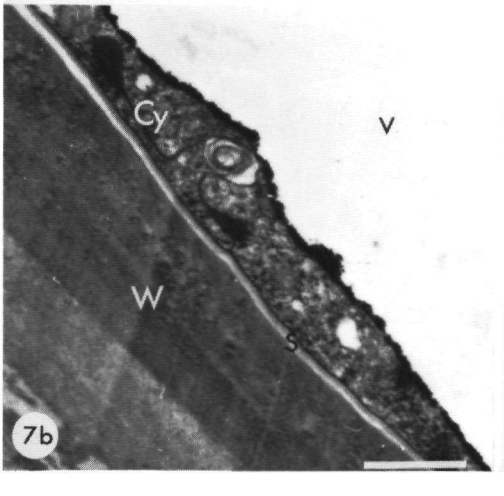
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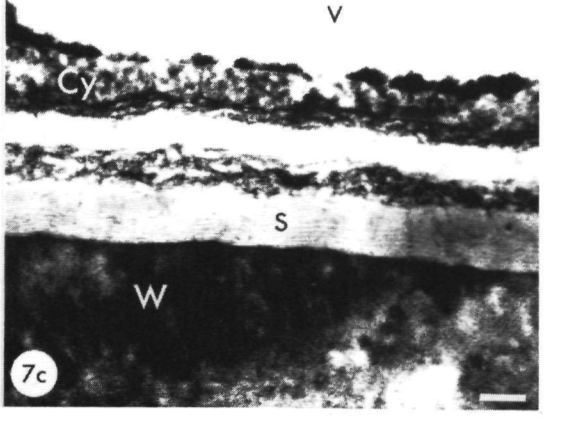
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side of the explants, because at these sides important events, like cell division and floral bud formation occurred (Wilms & Sassen 1987) At the apical part of the explants eventually some callus was formed

Four days after explantation distinct rows of cells were formed, resulting from periclinal cell divisions A periderm-like structure was formed Using fluorescence microscopy first signs of autofluorescence were found in the cortex cells most close to the medium (Fig 3) Sometimes granular deposits were observed in the intercellular spaces (data not shown) Two types of cells were formed at the central side of the explants cells with autofluorescence ("suberin-cells") and cells with no autofluorescence ('callus-cells") (Fig 3) At the most basal side of the explant irregular cell divisions occur

After 7 days of culture more periclinal cell division occurred at the central side of the explants Autofluorescence appeared in suberin-cells (Fig 4A B C) Using different dyes (Table 1) it could be shown that suberin is deposited in walls of the "suberin-cells' of the most central cortex cells Sudan III stained the 'suberin-cells , while 'callus-cells' were unstained (Fig 4D) At the basal side, no 'suberin-cells ' are found (Fig 5) Eventually, after 14 days of culturing, "suberin-cells" were observed at the basal side (Fig 6) In the electron microscope an electron lucid region was observed between the plasmamembrane and the cell wall in "suberin-cells" at the central side, with fine light and dark lamellation (Fig 7A,B C), indicating suberin deposition In "callus-cells" no such lamellae were detected (Fig 7D)

In order to distinguish between suberin, cutin and lignin deposition, the absorbtion spectra were measured from different parts of the explants, with the method described by Willemse (1981) The peak of the absorbtion spectrum for the cells at the central side of the explants was 515 nm, for the cutin layer at the epidermis 510 nm and 465 nm for the tracheary elements (lignin is deposited in cell walls during tracheary element formation (Roberts 1976)) (Fig 8) During the examination with ultraviolet light the fading of the autofluorescence of the cells at the central side of 4 days old explants was rapid which indicated that small quantities of suberin were deposited In 7 days old explants fading was hardly detectable

On control medium, explants reacted in a similar way as on bud inducing medium Four to seven days after explantation rows of cells were

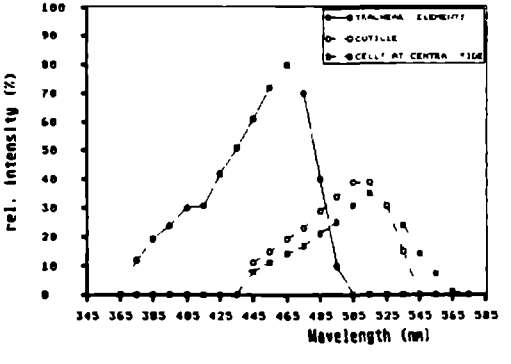

Fig 8 Absorbtion spectra of three different regions of a 7 d old explant after illumination of the explants with ultraviolet light (using the method of Willemse 1981)

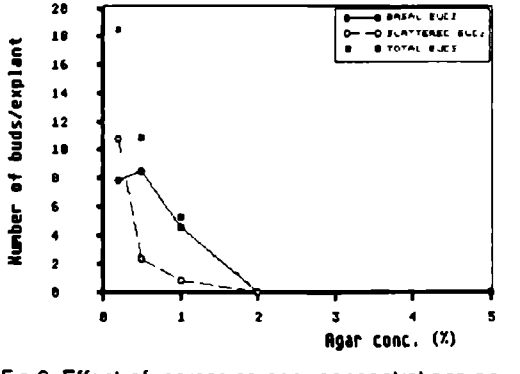

Fig 9 Effect of increasing agar concentrations on floral bud formation Explants were cultured on bud inducing medium (10^{-7}M NAA, 10^{-6}M BAP) with different concentrations of agar Basal and scattered bud formation was determined after 14 d of culture For each experiment 20 explants were used

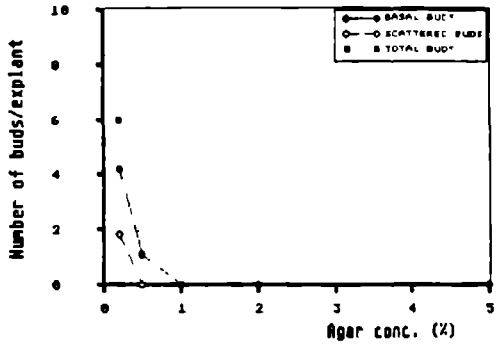

Fig 10 Effect of increasing agar concentrations on floral bud formation Explants were cultured on control medium (10^{-7}M NAA, 10^{-7}M BAP) with different concentrations of agar Basal and scattered bud formation were determined after 14 d of culture For each experiment 20 explants were used

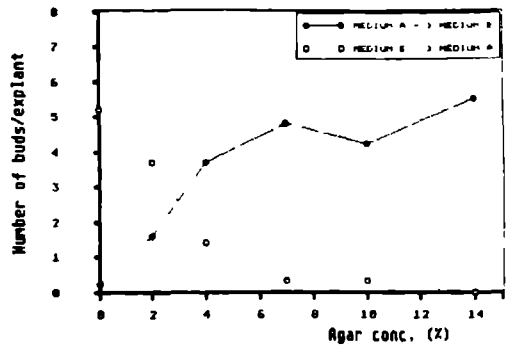

Fig 11 Sensitivity of explants to hormones Explants were cultured on bud inducing medium (medium A) and transferred to control medium (medium B) after 2, 4, 7 and 10 days Other explants were cultured on medium B and transferred to medium A after 2 4, 7 and 10 days O days on medium A means 14 days on medium B

Medium A M&S containing 10^{-6}M NAA, 10^{-7}M BAP, 125 mM glucose and 1 % agar

Medium B M&S containing 10^{-7}M NAA, 10^{-7}M BAP, 125 mM glucose and 1 % agar

formed and an autofluorescent layer was identified in cells adjacent to the medium However no "callus-cells' were formed at the basal side of the explants

Contact with Medium.

Explants cultured on medium with a high concentration of agar (2% and 5% agar) did not form buds even after 14 days of culturing (Fig 9) Hardly any cell divisions occurred at the central side of the explants With decreasing agar concentration of the medium the number of buds per explant increased Not only the number of buds changed but also their location on the explants On explants cultured on 1% agar buds were formed only at the basal side of the explants Explants cultured on medium with 0 5% and 0 2% agar also developed buds on the rest of the explants The scattered buds were nearly always located at the edges of the explants

Explants cultured on medium with 5% agar hardly showed any cell division When stained with phloroglucinol-HCl and illuminated with ultraviolet light, a thick autofluorescent layer was visible at the central side No "callus-cells" were observed Whenever explants were cultured on medium with less agar, more "callus-cells' were formed On medium with 0 2% agar no "suberin-cells" were observed

On control medium with low concentrations of agar (0 2% and 0 5% agar) floral buds were formed (Fig 10), indicating that sufficient hormones are available to induce buds

Sensitivity of Explants to Hormones

Explants cultured for 2 days on bud inducing medium and transferred for 12 days on control medium hardly developed any buds Four days on a bud inducing medium was required to develop a number of buds on the explants equal to explants cultured for 14 days on bud inducing medium (Fig 11)

Explants cultured for more than 4 days on control medium failed to develop buds when transferred to bud inducing medium When the explants were transferred from control to bud inducing medium before the 4the day of culturing, a normal number of buds was formed (Fig 11)

DISCUSSION

The first light microscopically visible reaction of the cortex cells adjacent to the cut surface is cell division, preceded by a re-orientation of cortical microtubules (Wilms & Derksen 1988) As was observed previously, the division plane of the new cell walls is parallel to the cut surface (Wilms & Sassen 1987) This was also observed in tissue strips of *Nautilocalyx lynchii* (Venverloo *et al* 1980) and in cuttings of *Agasthis australis* (White & Lovell 1984) In tobacco, within 4 days after wounding several rows of new cells are formed at the central side of the explants, resulting from longitudinal cell divisions At the basal side the first cell divisions occur also parallel to the cut surface After 4 days of culturing callus cells develop at this side from irregular cell divisions

From our experiments it can be concluded that no lignin is deposited in cells at the central side of the explants From light and fluorescence microscopical studies it is hardly possible to distinguish between suberin and cutin However their localization within the cell is different Cutin is deposited in the outer part of the cell wall exclusively and suberin in close association with the plasmamembrane (Frey-Wyssling 1976) In cells at the central side of the explants fine light and dark lamellations between the cell wall and the plasmamembrane were observed under the electron microscope This characteristic lamellation was also observed by Ryser *et al* (1983) and identified as suberin We therefore conclude that suberin is deposited in the cells at the central side of the explants

In tobacco explants cultured on medium with 1% agar, suberization starts 4 days after wounding The first changes occurred within 24 hours after wounding As in Geranium cuttings, granular suberin was deposited in intercellular spaces (Cline & Neely 1983) In tobacco explants, 4 to 7 days after wounding a suberin layer is deposited between the plasmamembrane and the cell wall in cells at the central side of tobacco explants creating an impervious layer The rapid fading of the autofluorescence of the cells at the central side of 4 day old explants indicate that the suberin layer is very thin Seven days after explantation a thick layer is deposited and fading was hardly detectable We did not find a lignification of the cells prior to suberization, as was reported for various plant species and organs by Kolattukudy (1984) and Rittinger et al (1987)

Between these 'suberin-cells" "callus-cells are formed that did not contain any suberin Via these "callus-cells' water and hormone uptake probably takes place In a dry environment (medium with 5% agar) hardly any cell divisions occurred suberin is deposited in all cells at the central side of the explants and no 'callus-cells arrise No floral buds were formed on these explants In an environment with a higher accessibility for water and hormones less suberin containing cells are formed and more callus-cells It seems that these explants can take up more hormones and consequently buds can be formed on the explant The number and location of buds formed on fluid medium (0 2% agar) is comparable to explants cultured on solid medium (1% agar) with a high concentration of NAA (10^{-6}M NAA) (Van den Ende *et al* 1984, Smulders *et al* 1988) In medium with low agar concentration more hormones become available to the explants and more floral buds may be induced

Our results show that when explants were cultured on bud inducing medium with 1% agar a period of 4 days on this medium was sufficient for optimal bud formation This was also reported by Smulders et al (1988) After this period, transfer to control medium had no effect on total number of buds formed In explants cultured on control medium with 1% agar for 2-4 days no meristem-like centres occurred at the basal side, at places where floral buds were expected to develop (Wilms & Sassen 1987) Whenever these explants were transferred to bud inducing medium, floral

buds developed after 10–12 days Therefore the tissue was still sensitive to hormones However, an increased period of culture on control medium made the tissue insensitive and no floral buds developed 14 days after explantation Although we can not exclude the possibility that the sensitivity of the cells may be changed by changes in ploidy level of the nuclei of the cells after explantation (Smith & Street 1974), we conclude that the explant can not take up enough hormones to induce floral buds because of the suberin layer formed after 4–7 days after explantation, acting as a barrier for hormone uptake

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REFERENCES

Biggs AR (1984) Boundary-zone formation in peach bark ion response to wounds and *Cytospora leucostoma* infection Can J Bot 62 2814–2821

---- (1985) Detection of impervious tissue in tree bark with selective histochemistry and fluorescence microscopy Stain Technol 60 299–304

---- (1986) Phellogen regeneration in injured peach tree bark Ann Bot 57 463–470

---- Northover J (1985) Formation of the primary protective layer and phellogen after leaf abscission in peach Can J Bot 63 1547–1550

Cline MN Neely D (1983) The histology and histochemistry of wound-healing process in geranium cuttings J Amer Soc Hort Sci 108 496–502

Eastman PAK Dengler NG, Peterson CA (1988) Suberized bundle sheaths in grasses (*Poaceae*) of different photosynthetic types I Anatomy, ultrastructure and histochemistry Protoplasma 142 92–111

Frey-Wyssling A (1976) The Plant Cell Wall In Encyclopedia of Plant Anatomy vol 3 Part 4, p 41 Gebruder Borntraeger,Berlin–Stuttgart

Gunning BES (1982) The cytokinetic apparatus its development and spatial regulation In Lloyd, C W (ed) The cytoskeleton in plant growth and development Chapter 11 pp 229–292 Academic Press, London

Gurr E (1956) A practical manual of medical and biological staining techniques Interscience Publishers, New York

Imaseki H (1985) Hormonal control of wound-induced responses In Pharis, R P & Reid D M (eds) Encyclopedia of Plant Physiology Volume 11 Hormonal regulation of Development III pp 485–512

Jensen WA (1962) Botanical Histochemistry Freeman, N H and Co , San francisco

Kolattukudy PE (1981) Structure, biosynthesis and biodegradation of cutin and suberin Annu Rev Plant Physiol 32 539–567

---- (1984) Biochemistry and function of cutin and suberin Can J Bot 62 2918–2933

Lipetz J (1970) Wound-healing in higher plants Int Rev Cytol 27 1–28

Murashige T Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture Physiol Plant 15 373–397

Rittinger PA Biggs AP Peirson DR (1987) Histochemistry of lignin and suberin deposition in boundary layers formed after wounding in various plant species and organs Can J Bot 65 1886–1892

Roberts LW (1976) Cytodifferentiation in plants xylogenesis as a model system Cambridge University Press Cambridge

Ryser U, Meier H Holloway PJ (1983) Identification and localization of suberin in the cell walls of green cotton fibres (*Gossypium hirsutum* L var green lint) Protoplasma 117 196–205

Smith SM, Street HE (1974) The decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota* L) are serially subcultured Ann Bot 38 223–241

Smulder MJM Janssen GFE, Croes AF, Barendse GWM, Wullems GJ (1988) Auxin regulation of flower bud formation in tobacco explants J Exp Bot 39 451–459

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy J Ultrastr Res 26 31–34

Van den Ende G, Croes AF, Kemp A, Barendse GWM Kroh M (1984) Floral morphogenesis in thin-layer tissue cultures of *Nicotiana tabacum* Physiol Plant 62 83–88

Venverloo CJ, Hovenkamp PH, Weeda AJ Libbenga KR (1980) Cell division in *Nautilocalyx*

explants I Phragmosome, preprophase band and plane of cell division Z Pflanzenphysiol 100 161–174

White J, Lovell PH (1984) Anatomical changes which occur in cuttings of *Agathis australis* (D Don) lindl I Wounding responses Ann Bot 54 621–632

Willemse MTM (1981) Changes in autofluorescence of lignin In Robinson DG Quader H (eds) Cell wall 1981 pp 242–250 Wissenschaftlicher–Verlag, Stuttgart

Wilms FHA, Derksen J (1988) Reorganization of cortical microtubules during cell differentiation in tobacco explants Protoplasma 146 127–132

----, Sassen MMA (1987) Origin and development of floral buds in tobacco explants New Phytol 105 57–65

III. CORTICAL MICROTUBULES IN CORTEX CELLS OF TOBACCO EXPLANTS

CHAPTER 4.

Reorganization of cortical microtubules during cell differentiation in tobacco explants

Protoplasma (1988) 146 127-132

PROTOPLASMA

Reorganization of Cortical Microtubules During Cell Differentiation in Tobacco Explants

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Summary

Using indirect immunofluorescence on polyethylene glycol embedded material, the organization of cortical microtubules (MTs) has been studied in explants of *Nicotiana tabacum* Within 6 hours after explantation the orientation of the cortical MTs shifts from transverse to longitudinal to the long axis of the cell in all cells This change of direction is followed by further shifts that occur only locally and predict the orientation of future cell divisions These reorientations are independent of the formation of protrusions and buds that will develop in the explants (after 4 7 days) and they represent a stage of de-differentiation of the explants
After two days of culturing clusters of cells can be recognized, at the proximal side of the explants, with randomly oriented cortical MTs These regions represent the origin of the protrusions from which floral buds will develop The formation of these clusters represent the first signs of re-differentiation and formation of new polar axes in the explants The cells thus show a very early commitment (within 2 days) as to their differentiation

Keywords Cell differentiation, Microtubules, Tissue culture, Tobacco

Abbreviations BAP benzyl-amino-purine DMSO dimethylsulfoxid, EGTA ethylene glycol bis(2 aminoethylether)-N,N,N' N'-tetraacetic acid, GA glutaraldehyde, MTs microtubules, MTOCs microtubule organizing centres, NAA α-naphthalene acetic acid, PEG polyethylene glycol, PFA paraformaldehyde, PPBs preprophase bands

1. Introduction

Using *in vitro* culture techniques organogenesis can be studied under highly defined conditions in many plant systems Even differentiated tissues, when brought into culture under proper conditions, develop into entire new organs (Tran Thanh van 1977, Williams and Maheswaran 1986) In plants, morphogenesis occurs by directed cell divisions followed by a polar cell expansion leading to marked polarity of cells, tissues and organs In both cell division and cell polarity cortical microtubules (MTs) play an important role (review Green 1980) place and plane of the new cell walls are predicted by preprophase bands (review Dustin 1984) and cell expansion occurs transverse to the direction of the cortical MTs (Gunning and Hardham 1982, Traas *et al* 1984) Though cortical MTs at interphase and preprophase bands (PPBs) may show corresponding orientations, no clear evidence is available concerning their mutual relations
Tissue strips from floral stalks of *Nicotiana tabacum* develop entire flowers when explanted on an appropriate medium (Tran Thanh van 1977, van den Ende *et al.* 1984) The number and place of buds formed depend on conditions of both explants and medium (van den Ende *et al* 1984, Wilms and Sassen 1987). In such explants the original polarity is lost and replaced by new polar axes (Wilms and Sassen 1987) The first cell divisions occur near the cut surfaces followed by the formation of meristem like centres in the area where flower buds will develop The orientation of the first divisions is parallel to the cut surface, in the meristem like centres the orientations are random (Wilms and Sassen 1987). Here we studied the changes in the microtubular skeletons after explantation, especially the relation between the interphase cortical MTs and the orientation of the new division plane at the surface and in the meristem like centres The results are discussed with respect to the loss of original polarity

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and the relation between interphase cortical MTs and orientation of the new division planes

2. Materials and Methods

2.1 Preparation and Culturing

Tissue strips 0.6 by 7 mm, were cut from floral stalks of *Nicotiana tabacum* as previously described by Wilms and Sassen (1987) and explanted on a solid Murashige and Skoog (M&S) medium (Murashige and Skoog 1962) supplied with 10 ^{7}M NAA (α-Naphthalene acetic acid) 10 ^{6}M BAP (Benzyl-amino purine) and 125 mM glucose. Under these conditions buds are formed only at the basal side proximal to the main axis of the explant. For more details concerning origin of the explants, polarity and terminology see Wilms and Sassen (1987). As a control, tissue strips were explanted on a solid M&S medium supplied with 10 ^{7}M NAA, 10 ^{7}M BAP and 125 mM glucose. On this medium no buds were formed. In order to examine the possible influence of the osmolality of the medium on the direction of cortical MTs in the cells we varied the glucose concentration from 25 mM to 125 mM glucose.

2.2 Light Microscopy

To follow the development of the explants they were fixed, sectioned and photographs were taken as previously described by Wilms and Sassen (1987). From these photographs drawings were made true to nature.

2.3 Immunocytochemistry

To study the microtubules in the cells, the explants were fixed in 4% paraformaldehyde (PFA) and 0.25% glutaraldehyde (GA) in a microtubule (MT) stabilizing buffer for 2 hours at room temperature. The MT stabilizing buffer contained 50 mM phosphate buffer, 10 mM EGTA, 5 mM $MgSO_4$ (see also Wick *et al* 1981) and 10% DMSO (Schroeder *et al* 1985). After rinsing in buffer preparations were embedded in aqueous polyethylene glycol (PEG) according to Hawes *et al* (1983). We used PEG 1500 as an embedding medium. 15 µm thick longitudinal sections were cut with a steel knife on a Reichert hand microtome and attached to poly-L lysine coated coverslips. In order to remove the PEG from the coverslips the preparations were carefully rinsed in water. coverslips were placed vertically in water for a few seconds and air dried. This procedure was repeated several times. The primary antibody, a monoclonal anti tubulin (MAS 076 Sera Labs) was added for 2–18 hours at 30 °C. The coverslips were carefully rinsed with buffer. The second antibody, a goat fluorescein isothyocyanate (FITC)— labelled anti-rat immunoglobulin G (IgG) (Nordic Labs BV, Tilburg, the Netherlands) was added, for 2 hours at 30 °C. The coverslips were rinsed again and mounted on Gelvatol with 2% propylgalate. Preparations were examined under a Leitz Orthoplan microscope with a 100× neofluar objectiens. In order to lower the fluorescence of the chloroplasts we used a green filter. Photographs were taken with a Leitz Vario Orthomat combination on Kodak Tri X film 400 ASA. Using sections, only part of the cortical plasma and no whole cells can be seen due to the large diameter of the cells (approximately 30 µm). When cell strips were used instead of sections (Traas *et al* 1984), similar results were obtained, however since the connections between the cells were lost only individual cells could be studied. Therefore we preferred to use sectioned material.

The direction of MTs in single cells was determined using a protractor in the ocular of the microscope. Four classes of directions of microtubules were distinguished: 0 to 30°, 30 to 60° and 60 to 90° to the cell axis and random (after Lang, Selker and Green 1984). Only cortical cells were investigated since the first cell divisions took place in the cell layers underneath the epidermis (Wilms and Sassen 1987). Moreover microtubules in epidermal cells were sometimes hardly visible.

To study changes in cell diameter, 10 µm cross sections were made from PEG embedded material. The cell-area was measured using a Kontron Videoplan computer.

3. Results

At the onset of the experiment nearly all cells had cortical MTs oriented transverse to the long axis of the cells (Fig 1*a*). Within 6 hours after explantation on bud forming medium the orientation of the MTs in the cells changed, first to mainly an oblique orientation (Fig 1*b*) followed by a further change till almost all cells showed longitudinally oriented MTs (Fig 1*c*). Hardly any cells had randomly oriented MTs (Fig 1*d*). Measurements of MT orientations in large numbers of cells from at least 5 different experiments are summarized in Fig 2. These changes occurred over the whole explants. Though no quantitative measurements of MT density could be made, no clear differences in MT density in the cells was observed during the experiments.

Sometimes different parts of the cells showed more than one major direction of MTs or MT-bundles. Cells with both transversely and obliquely oriented MTs were observed mainly during the first 2 hours of the experiment (Fig 3*a*), cells with MTs in both oblique and longitudinal directions were observed mainly between 2 and 6 hours after the onset of the experiment (Fig 3*b*). After the first 24 hours further changes in MT orientation were observed, but unlike the previous ones they occured only locally, resulting in four areas with different directions of MTs. These different areas are best seen in explants cultured for 4 days (Fig 4*b*, see also Fig 8 of Wilms and Sassen 1987). At the cut edges, region I at the basal side and region II at the central side (see for terminology Wilms and Sassen 1987) new cell walls are formed parallel to the wounded surfaces. Region III refers to the side where after 4 days meristematic centres occur. Region IV represents the rest of the explant where no visible changes are observed. The different regions are indicated in Fig 4*a* and *b*, drawn to nature from 2 and 4 days old explants. With the present immunofluorescence technique we clearly can identify region III within 48 hours of culturing. Within 36 hours after the onset of the experiments the cells of region I (Fig 4*a*) had almost entirely transverse to oblique oriented MTs (Fig 5, 36 {I}). Accordingly, in these cells PPBs, phragmoplasts and newly formed

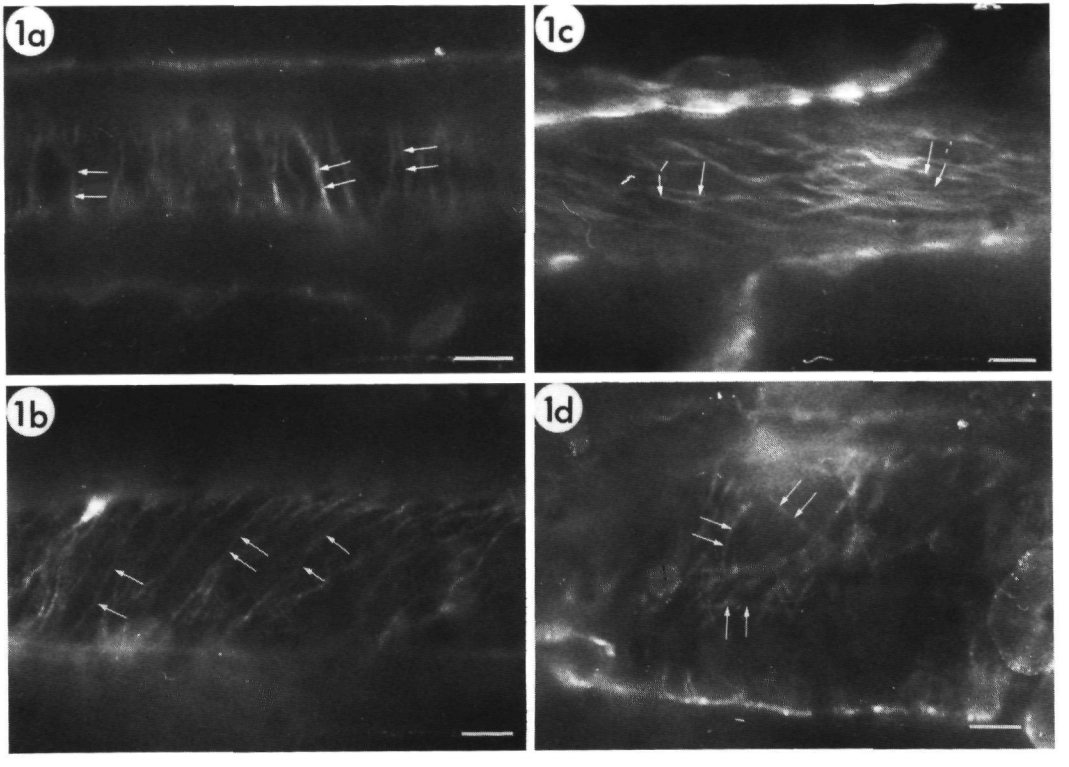

Fig. 1. Immunofluorescence microscopy of cortical cells of tobacco explants during the first 24 hours of culture on both bud forming and control medium, showing; *a* transverse, *b* oblique, *c* longitudinal, *d* random orientations of cortical microtubules with respect to the long axis of the cell. Arrows: microtubules. Bar: 5 µm. *a, d*: × 2,000, *b, c*: × 1,700

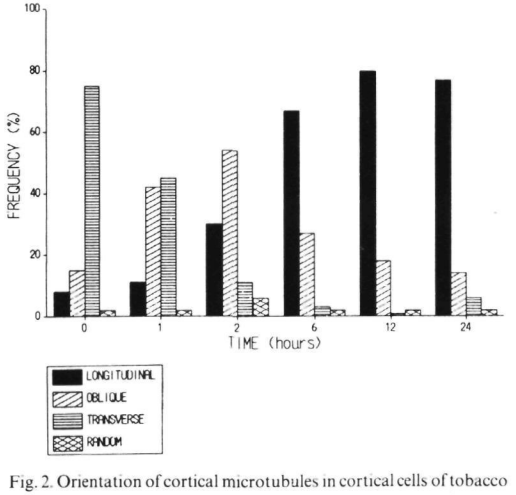

Fig. 2. Orientation of cortical microtubules in cortical cells of tobacco explants during the first 24 hours of culture on both bud forming and control medium. Number of cells: 400/time. S.D.: 1–15%

cell walls were formed transverse or oblique in respect to the cell axis, whereas chromosome separation occurred parallel to the cell axis. The cells of region II (Fig. 4*a*) (the cut surface in close contact with the medium) retained their longitudinal to oblique MTs (Fig. 5: 36.{II}). Also PPBs, phragmoplasts and newly formed cell walls were oriented parallel or oblique to the cell axis. The rest of the cells showed no changes. Between 36 and 48 hours after onset of the experiment, the cells of region III (Fig. 4*a*) showed further changes in MT orientation: relatively more cells were observed with randomly oriented MTs (Fig. 5: 48.{III}). Here no PPBs and phragmoplasts were observed and no cell divisions were found. At the other side of the explant, opposite to the basal side, no such regions could be observed.

The rest of the cortical cells of the explant (region IV in Fig. 4*a*) retained longitudinal oriented MTs (Fig. 5: 48.{IV}). Here also no PPBs, phragmoplasts and cell divisions occurred.

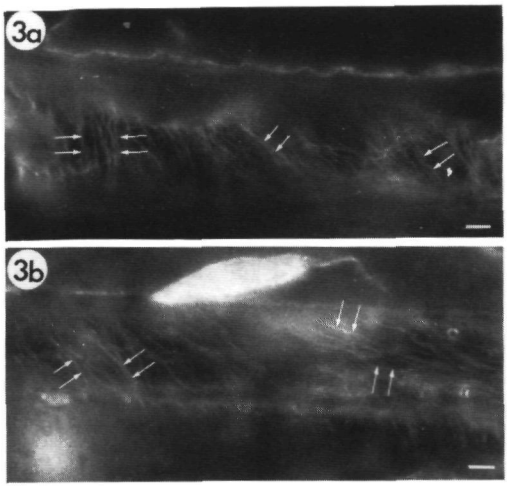

Fig. 3. Immunofluorescence microscopy of cortical cells showing; *a* transverse and oblique (between 0 and 2 hours of culturing), *b* oblique and longitudinal (between 2 and 6 hours of culturing) oriented microtubules with respect to the long axis of the cell, cultured in both bud forming and control medium. Arrows: microtubules. Bar: 5 µm. × 1,000

In explants on control medium, upon which no buds will develop, the direction of MTs was changed from transverse to longitudinal in all regions within six hours after the onset of the experiment. Between 1 and 2 days cortical MTs in cells of region I and region II were oriented transversely and longitudinally respectively. Cell divisions also occurred transverse and parallel to the cell axis respectively. However in cells of region III no changes were observed. Cells in region IV reacted the same as in cells cultured on bud forming medium. Variations of the glucose concentrations in the medium had no effect on the shifts of cortical MTs. Different glucose concentrations in the medium of the explants also had no effect on the number of buds formed after 14 days (6.5 buds per explant; compare WILMS and SASSEN 1987).

During the first 24 hours of the experiment no changes in cell diameter could be observed in explants cultured on both bud forming and control medium. After 48 hours of culturing the diameter of the cortical cells in region IV had increased 200%. No increase in the

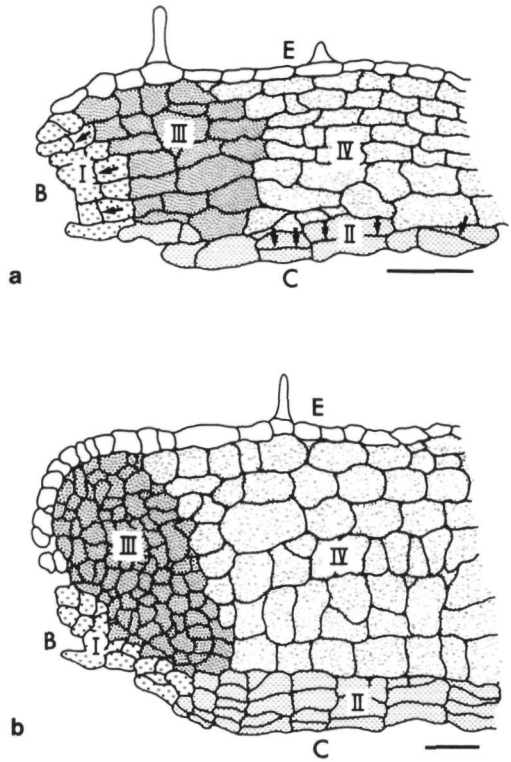

Fig. 4. Drawings of light microscopical longitudinal sections of explants cultured for; *a* 2 days, *b* 4 days on bud forming medium. Region *I* and region *II*: cut surfaces, region *III*: protrusion area, region *IV*: rest of explant. *B* Basal side, *C* central side, *E* epidermal side of explant. Arrows: newly formed cell wall. Bar: 0.1 mm. *a*: × 200, *b*: × 130

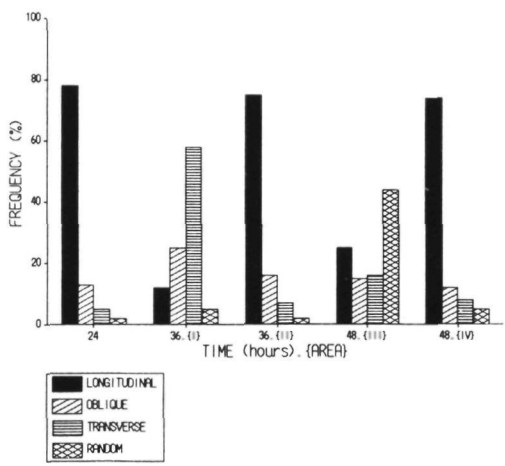

Fig. 5. Orientation of cortical microtubules in cortical cells of tobacco explants between 24 and 48 hours of culture on bud forming medium at different areas in the explants. *I* Basal side, *II* central side, *III* protrusion area, *IV* rest of explant. Number of cells: 100/area. S.D.: 1–15%

length of the cells during the first 48 hours of the experiment was observed

4. Discussion

The results clearly show that after explantation major changes occur in the organization of the cortical microtubular skeleton A first shift of 90° from transverse to longitudinal to the long axis of the cell occurs within 6 hours after explantation This shift occurs gradually in all cells and is followed by further changes which occur only locally and predict forthcoming cell divisions Major shifts in orientation of cortical MTs have been shown previously In mesenchym cells of *Zinnia elegans*, when cultured in a medium similar to that used here, cortical MTs change their orientation from longitudinal to transverse (Falconer *et al* 1985) These changes occur very slowly however (in the course of 7 days) and are part of the differentiation into xylem elements, whereas the changes in tobacco occur within a few hours and are part of a de-differentiation process (see below) Cortical MTs in cortex and epidermis cells of *Pisum sativum* and *Vigna radiata* change their orientation from transverse to longitudinal to the long axis of the cell within a few hours after increasing the osmolality of the medium (Roberts *et al* 1985) or after ethylene treatment (Steen and Chadwick 1981, Lang *et al* 1982, Roberts *et al* 1985) Since in tobacco explants the changes are independent of the glucose concentration in the medium changes in osmolality are probably not involved in the shifts in MT orientation Possibly, in tobacco, wound ethylene is involved in the first shift of MT orientation which precedes all other events However since the following shifts occur only locally, they can hardly be described as an overall response to wound-ethylene Changes of MT orientation can also occur from changes in cell volume In fibres of cotton the direction of cortical MTs originally is on average transverse to the cell axis During elongation the MTs change to a more helical arrangement (Quader *et al* 1987) Also changes in MT orientation of root cortex cells have been related to the direction of cell expansion at the cells surface (Traas *et al* 1984) However, the reorganization of the MTs in tobacco takes place some 18 hours before a change in cell diameter becomes obvious The shift of MT orientation precedes cell expansion, as is shown in the outer cortical and epidermal cells of *P sativum* and *V radiata* (Roberts *et al* 1985) Thus, if a causal relation exists cell expansion depends on the orientation of the cortical microtubular skeleton In *Cobaea* seed hairs cortical MTs change in orientation and density and become clustered, when cellulose deposition starts (Quader *et al* 1986) We did not observe any clustering of MTs in cortex cells of tobacco explants before, during or after the swelling of the cells

The mechanisms by which MTs change their orientation are still unknown It has been proposed that MTs could change their orientation via a cell division, *i e*, complete degradation and re establishment of new MTs (Lang Selker and Green 1984) This mechanism obviously cannot hold up for tobacco explants, because MTs change their orientation before cell division starts We cannot exclude the possibility that the entire microtubular helix changes its direction in a more or less intact form as proposed by Roberts *et al* (1985) and Lloyd (1986), or that new MT skeletons are formed while the old MT skeleton is still intact (Kirschner and Schulze 1986) The latter possibility may be supported by the occasional occurrence of two main orientations of MTs within a cell MTs are highly dynamic structures (Dustin 1984 Williams *et al* 1986), not only *in vitro* (Farrell *et al* 1987), but also *in vivo* (Kirschner and Mitchison 1986 Schulze and Kirschner 1987) During cell division cortical MTs change their orientation in the cell during re-establishment of cortical microtubular skeleton when they grow from microtubule organizing centers (MTOCs) at the nuclear envelope and reach the cell membrane (Wick and Duniec 1983) The MTOCs described at the cell membrane in *Azolla* by Gunning *et al* (1978) may represent such a case Thus shifts in MTs orientation in tobacco explants might occur by growth at one end of the MTs in a direction other than that of the original MTs It should be emphasized that the different possibilities mentioned are not necessarily mutually exclusive The driving force behind the reorganization of the MT skeleton is unknown Possibly other cytoskeleton elements, *i e*, actins, could be involved or MT reorganization might depend on changed gradients within the cell for example of Ca^{2+} Calmodulin (for discussion see also Quader *et al* 1986)

PPBs indicate the future division plane of the cell (Gunning and Hardham 1982) As shown here, prior to cell division the entire interphase MT skeleton changes direction parallel to the future division plane Striking in this respect is the occurrence of random cell divisions in regions containing cells with randomly oriented cortical MTs Thus a relation exists between the orientation of cortical MTs at interphase and the future division plane mediated by PPBs

Floral stalks represent a highly polar tissue an ex-

tended length axis and a rotational symmetry of the transverse axes are present Also, physiologically a certain polarity exists since under defined hormonal conditions buds will develop at the basal side of the explants (Wilms and Sassen 1987) Loss of polarity is a prerequisite if new organs are to be formed Since the cells of the stalks expand transverse to the orientation of the cortical MTs (see also above) the first shift of MT orientation in the explant should be taken as a sign of de-differentiation and loss of polarity This is very likely the case as this shift occurs in explants cultured on both bud forming and control medium The shift at the basal and the central side occur also on control medium and represent wound reactions, as they occur specifically at the wounded surface more or less independent of the hormonal conditions of the medium The randomization of the MTs of the cells in area's where primordia and buds will develop, however, depends on the hormonal conditions of the medium Between these area's and the future primordia new polar axes will develop (Wilms and Sassen 1987) and therefore this randomization should be taken as the first sign of re-differentiation Also *in vivo* randomization of MTs occurs before new polar axes become established (Lyndon *et al* 1982) Thus cells 36 hours after explantation when no other morphological signs of differentiation are visible, already show a commitment regarding this differentiation and the establishment of a new polar axis As in animal systems plant cells show an early commitment where MT reorganization may not be the first event

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References

Dustin P (1934) Microtubules Springer Berlin Heidelberg New York

Falconer MM Seagull RW (1985) Xylogenesis in tissue culture taxol effects on microtubule reorientation and lateral association in differentiating cells Protoplasma 128 157 166

Farrell KW Jordan MA Miller HP Wilson L (1987) Phase dynamics at microtubule ends the coexistence of microtubule length changes and treadmilling J Cell Biol 104 1035–1046

Green PB (1980) Organogenesis—a biophysical view Annu Rev Plant Physiol 31 51 82

Gunning BES Hardham AR (1982) Microtubules Annu Rev Plant Physiol 33 651–698

— Hughes JF (1978) Evidences for initiation of microtubules in discrete regions of the cell cortex in *Azolla* root tip cells and an hypothesis on the development of cortical arrays of microtubules Planta 143 161–179

Hawes C Juniper BE Horne JC (1983) Electron microscopy of resin free sections of plant cells Protoplasma 115 88–93

Kirschner M Mitchison T (1986) Beyond self assembly from microtubules to morphogenesis Cell 45 329 342

Schulze E (1986) Morphogenesis and the control of microtubule dynamics in cells J Cell Sci [Suppl] 5 293–310

Lang JM Fisher WR Green PB (1982) Effects of ethylene on the orientation of microtubules and cellulose microfibrils of Pea epicotyl cells with polylamellate cell walls Protoplasma 110 5–14

Lang Selker JM Green PB (1984) Organogenesis in *Graptopetalum paraguayense* E Walter shifts in orientation of cortical microtubule arrays are associated with periclinal divisions Planta 160 289–297

Lloyd CW (1986) Towards a dynamic helical model for the influence of microtubules on wall patterns in plants Int Rev Cytol 86 1–51

Lyndon RF (1982) Changes in polarity of growth during leaf initiation in the Pea *Pisum sativum* L Ann Bot 49 281–290

Murashige T Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture Physiol Plant 15 373–397

Quader H Deichgraber G Schnepf E (1986) The cytoskeleton of *Cobaea* seed hairs Patterning during cell wall differentiation Planta 168 1–10

Herth W Ryser U Schnepf E (1987) Cytoskeletal elements in cotton seed hair development *in vitro* their possible regulatory role in cell wall organization Protoplasma 137 56–62

Roberts IN Lloyd CW Roberts K (1985) Ethylene induced microtubule reorientation mediation by helical arrays Planta 164 439 447

Schroeder M Weiland J Weber K (1985) Immunofluorescence microscopy of microtubules in plant cells stabilization by dimethylsulfoxide Eur J Cell Biol 38 311–318

Schulze E Kirschner F (1986) Microtubule dynamics in interphase cells J Cell Biol 102 1020 1031

Steen DA Chadwick AV (1981) Ethylene effects in Pea stem tissue Evidence of microtubule mediation Plant Physiol 67 460 466

Traas JA Bryat P Derksen JW (1984) Changes in microtubule arrays during the differentiation of cortical root cells of *Raphanus sativus* Eur J Cell Biol 34 229–238

Tran Thanh van K (1977) Regulation of morphogenesis In Barz W Reinhard E Zenk MH (eds) Plant tissue culture and its bio technological applications Springer Berlin Heidelberg New York pp 367 385

Van den Ende G Croes AF Kemp A Barendse GWM Kroh M (1984) Development of flower buds in thin layer tissue cultures of *Nicotiana tabacum* Physiol Plant 62 83 88

Wick SM Duniec J (1983) Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells I Preprophase band development and concomitant appearance of nuclear envelope associated tubulin J Cell Biol 97 235 243

— Seagull RW Osborn M Weber K Gunning BES (1981) Immunofluorescence microscopy of organized microtubule arrays in structurally stabilized meristematic plant cells J Cell Biol 89 685–690

Williams EG Maheswaran G (1986) Somatic embryogenesis Factors influencing coordinated behaviour of cells as an embryogenic group Ann Bot 57 443–462

Williams RC Jr Caplow M McIntosh Jr (1986) Cytoskeleton Dynamic microtubule dynamics Nature 324 106 107

Wilms FHA Sassen MMA (1987) Origin and development of floral buds in tobacco explants New Phytol 105 57–65

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CHAPTER 5.

INVOLVEMENT OF CORTICAL MICROTUBULES IN CELL POLARITY, ORIGIN OF NEW POLARITY AXES AND XYLOGENESIS DURING FLORAL BUD FORMATION IN TOBACCO EXPLANTS.

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SUMMARY

The organization of cortical microtubules was studied in relation to floral bud formation and the establishment of new polarity axes in tissue strips of tobacco explants. Four days after explantation, protrusions are formed from meristem-like centres at the proximal side of the explants. During the next three days, several events occur parallel and sequential, resulting in the formation of floral primordia.

At the perifery of the protrusions, patches of cells are formed after 4 days of culturing. In these cells, the orientation of cortical microtubules is transverse to the longitudinal axis of the surface of the protrusion. Anticlinal cell divisions occur, as one of the first signs of floral primordia formation.

At the same time cortical microtubules in cells inside the protrusion are oriented in circular or ellipsoid bands. Cell wall material is deposited in association with these microtubule bands and tracheary elements are formed, resulting in the formation of tracheary centres.

Between the patches in the epidermis with cell divisions and the tracheary centre, cells with cortical microtubules oriented transverse to the longitudinal axis of the cell are found, forming new polarity axes. After some time tracheary elements are formed which connect the floral primordia with the tracheary centre.

The role of cortical microtubules in floral bud formation and xylogenesis, and the development of new polarity axes is discussed.

INTRODUCTION

Organogenesis can be studied under experimentally controlled conditions, using *in vitro* plant systems (Tran Thanh Van 1977, Williams & Maheswaran 1986). Cultured under proper conditions it is possible to determine location and number of buds formed on explants of floral stalks of *Nicotiana tabacum* (Tran Thanh Van 1973, Wilms & Sassen 1987). After 2 days of culturing meristem-like centres are formed at the basal side of the explant, proximal to the main axis (Wilms & Derksen 1988). Within 4 to 7 days, these centres develop into protrusions, tracheary elements inside the meristem and flower primordia at the perifery are formed (Wilms & Sassen 1987).

The formation of new organs requires changes in cell polarity (reviews: Green 1980, Hardham 1982, Lyndon 1982). Marked factors accompanying the formation of new polarity axes in cells and tissues include changes in the orientation of cortical microtubules (MTs) and cellulose microfibrils (CMFs). MTs predict place and plane of the new cell walls, by forming preprophase bands (Gunning & Hardham 1982). CMF orientation in cell walls determines the direction of cell expansion (Hardham 1982).

Differentiation of meristems into different cells

and tissues can be examined by using immunocytochemical techniques for MT detection (Sakaguchi *et al* 1988 Wilms & Derksen 1988)

Xylogenesis can be studied *in vivo* (Goosen-de Roo 1973, Hardham & Gunning 1979, Sachs 1981), in cell cultures (Falconer & Seagull 1985) and in tissue cultures (Tran Thanh Van 1977, Wilms & Sassen 1987) by means of electron microscopy and fluorescence microscopy

The purpose of this study is to examine floral bud formation and to demonstrate the involvement of cortical MTs in cell polarity, in origin of new polarity axes and in tracheary element formation (xylogenesis)

MATERIALS AND METHODS

Preparation and Culturing

Tissue strips, 0 6 by 7 mm, were cut from floral stalks of *Nicotiana tabacum* as previously described by Wilms and Sassen (1987) and explanted on a solid Murashige and Skoog medium (Murashige & Skoog 1962) supplied with 10^{-7}M NAA (1-Naphthalene acetic acid), 10^{-6}M BAP (Benzyl-amino-purine) 125 mM glucose and 1% agar Under these conditions, after 7 to 14 days buds will develop at the basal side proximal to the main axis of the explant For more detail concerning origin of the explants polarity and terminology see Wilms and Sassen (1987)

Immunocytochemistry

Explants were fixed in 4% paraformaldehyde in a microtubule (MT) stabilizing buffer for 2 hours at room temperature and embedded in aqueous polyethylene glycol (PEG) 1500 as described by Wilms and Derksen (1988) The MT stabilizing buffer contained 50 mM phosphate buffer, 10 mM EGTA, 5 mM $MgSO_4$ and 10% DMSO Longitudinal sections (5 μm thick) were cut with a steel knife on a Reichert hand microtome and attached to poly-L-lysine coated coverslips MTs were visualized using indirect immunofluorescence with a monoclonal anti-tubuline (MAS 077b, Sera Labs) as the primary antibody and goat fluorescein isothyocyanate(FITC)-labelled anti-rat immunoglobulin G (IgG) (Nordic Labs BV Tilburg the Netherlands) as the second antibody The sections were mounted on Citifluor and examined under a Leitz varia orthomat combination, with appropriate illumination and filters for FITC 450-490 nm excitation filter and 515 nm barrier filter)

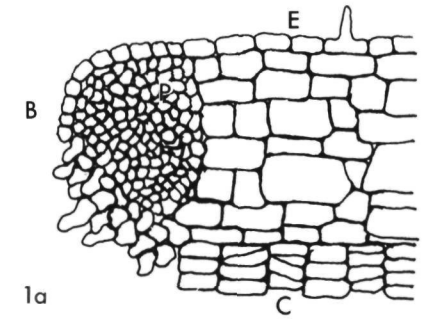
Lignin deposition is one of the first signs of differentiation into tracheary elements (Roberts 1976) Deposition of lignin was studied by means of its autofluorescence under ultraviolet light illumination (355-425 nm excitation filter 460 nm barrier filter)

From photographs of sections drawings were

Fig 1 A) Drawing true to nature of light microscopic image of a longitudinal section of a 4 d old explant showing the basal side of the explant with a protrusion area Rectangles in drawing refer to Fig 1B-E
B C) Immunofluorescence microscopy of epidermal cells showing longitudinal and random orientations of cortical MTs respectively
D,E) Immunofluorescence microscopy of cells inside a protrusion, showing random orientations of cortical MTs

Fig 2 A) Drawing true to nature of light microscopic image of a longitudinal section of a 5 d old explant showing the basal side of the explant with a protrusion area Rectangles in drawing refer to Fig 2B-E
B) Immunofluorescence microscopy of epidermal cells showing transverse orientations of cortical MTs
C) Immunofluorescence microscopy of cells inside the protrusion with ellipsoid patterns of MTs
D) Same cells as in Fig 2C examined under ultraviolet light No autofluorescence was observed
E) Immunofluorescence microscopy of cells inside a protrusion, showing random orientations of cortical MTs

B basal side C central side, E epidermal side, P protrusion, arrow MT, arrowhead ellipsoid pattern of MTs Bar 10 μm



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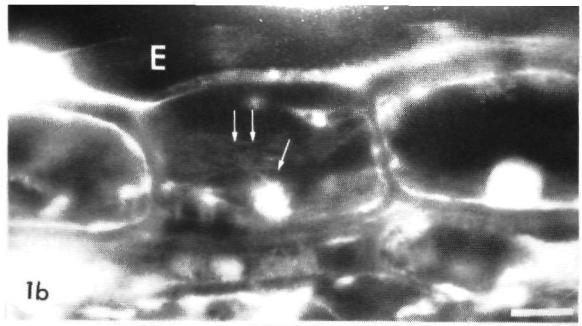

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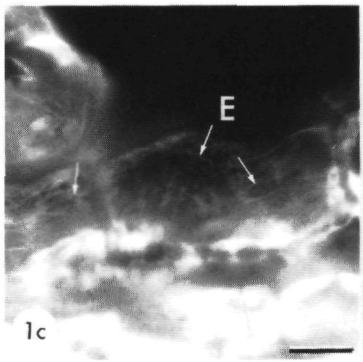

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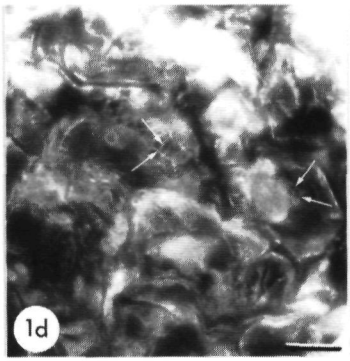

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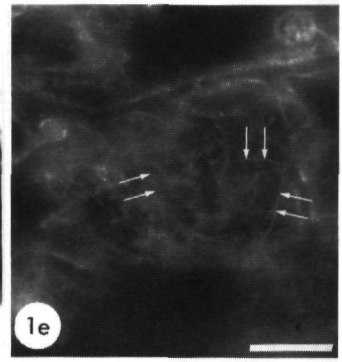

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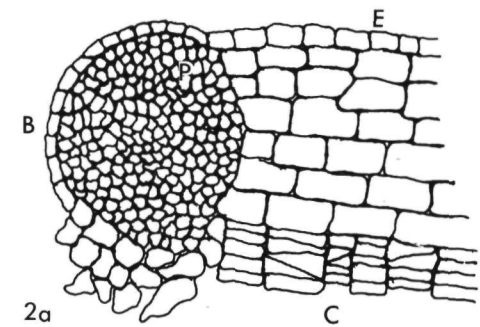

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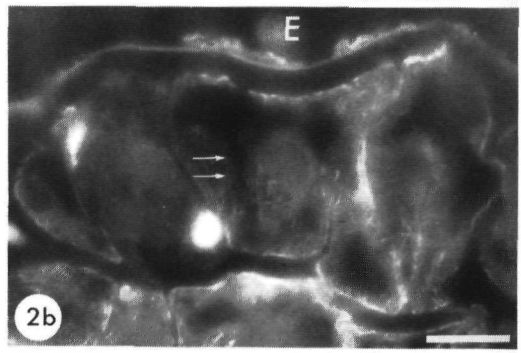

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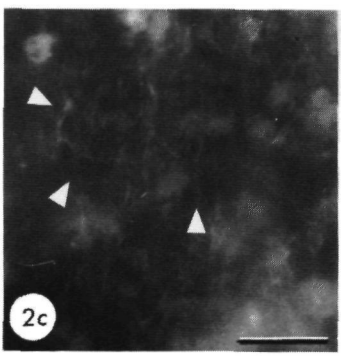

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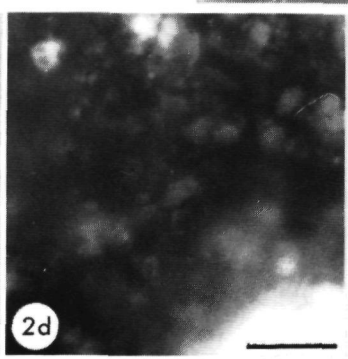

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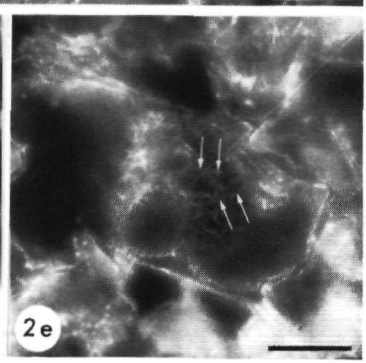

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made true to nature

RESULTS

Floral bud development is a complex event several processes occur simultaneously, and growth does not occur synchroneously However, bud development can be inferred from serial sections of a number of explants Generally, bud formation starts 4 days after explantation and is completed after 14 days of culturing (Wilms & Sassen 1987)

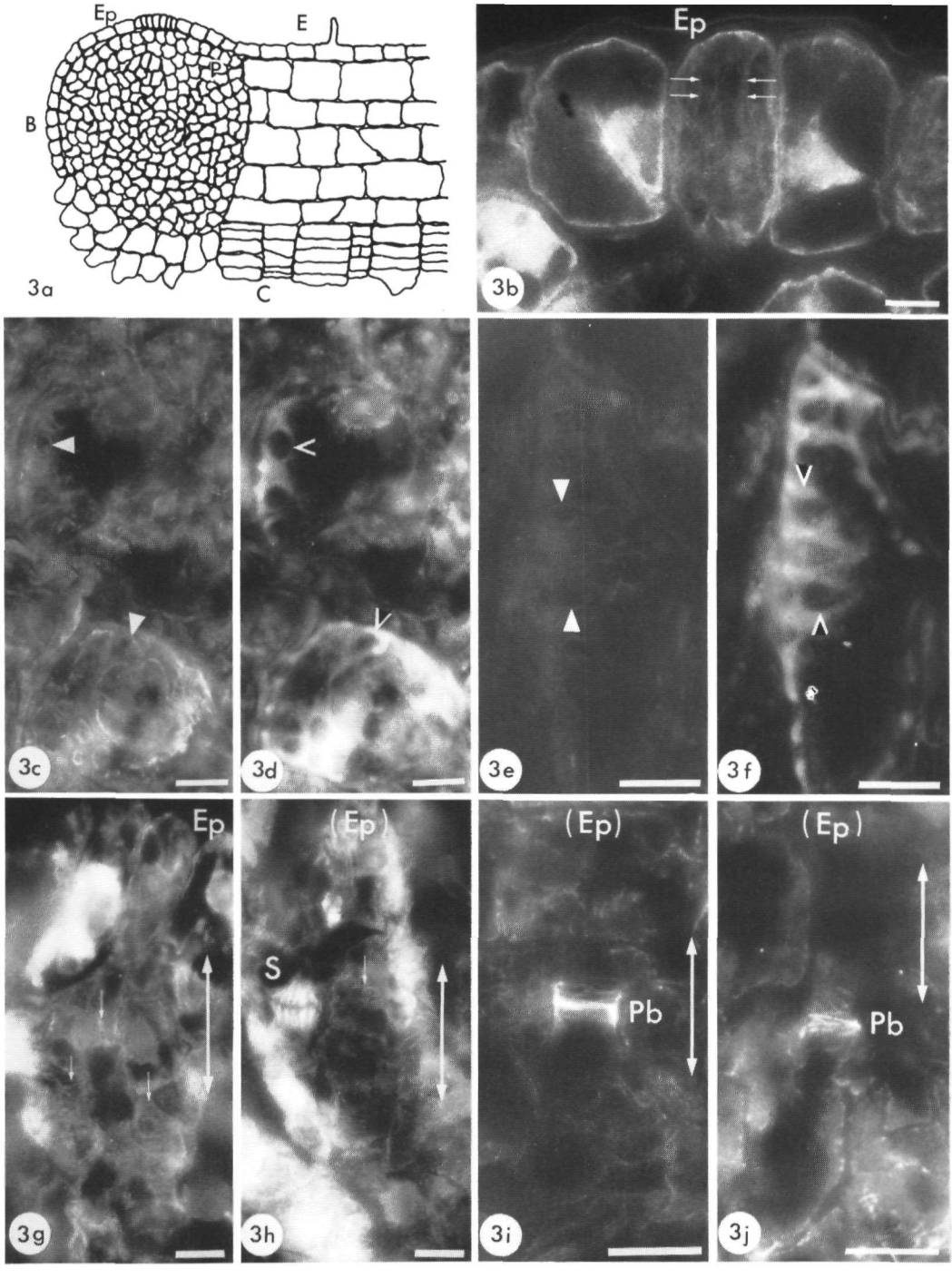
After 4 days of culturing meristem-like centres (= protrusions) had developed at the basal side of the explant (Fig 1A) In the epidermis, cells with cortical MTs oriented randomly or parallel to the longitudinal axis of the surface of the protrusion could be observed (Fig 1B C) No cell divisions were seen In the remaining cells of the protrusions the orientation of cortical microtubules was mostly random (Fig 1D,E) Sometimes cells showed parallel alligned cortical MTs

In five day old explants epidermal cells at distinct patches at the perifery of the protrusion showed MTs oriented transverse to the surface of the protrusion (Fig 2B) Other cells showed random orientations Cells inside the protrusion showed characteristic patterns of cortical MTs, with parallel to circular or ellipsoid configurations (Fig 2C) No fluorescence could be detected in these cells with ultraviolet light (Fig 2D) thus no lignin was deposited Other cells inside the protrusion had randomly oriented MTs (Fig 2E)

Six days after explantation anticlinal cell divisions were observed in cells at the patches of the epidermis with transverse oriented cortical MTs (Fig 3B) Tracheary element formation could be detected unambiguously by means of autofluorescence under ultraviolet light (Fig 3D,F) *This autofluorescence was always associated* with the circular or ellipsoid patterns of MTs (Fig 3C E) The tracheary elements were grouped into a tracheary centre In cells just beneath the patches of division at the epidermis, more cell divisions occurred Between the tracheary centres and the region of epidermal cell divisons cells extended These cells showed MTs oriented transverse to the longitudinal cell axis (Fig 3G H) and preprophase bands in the same orientation were visible (Fig 3I,J)

On seven day old explants the first morphological signs of floral primordia at the surface of the protrusions were visible (Fig 4A) The epidermal cells of the new outgrowth had divided (Fig 4B) and in the new outgrowth new meristematic regions had developed From the tracheary centre inside the protrusion, tracheary element reached allmost till this meristematic region (Fig 4C,D) These floral primordia later developed into entire new flowers as described for *in vivo*

Fig 3 A) Drawing true to nature of light microscopic image of a longitudinal section of a 6 d old explant showing the basal side of the explant with the protrusion area Rectangles in drawing refer to Fig 3B–J
B) Immunofluorescence microscopy of patches of epidermal cells showing transverse orientations of cortical MTs
C,E) Immunofluorescence microscopy of cells inside the protrusion with circular and ellipsoid patterns of MTs
D,F) Same cells as in Fig 3C,E examined under ultraviolet light Tracheary cells with autofluorescence were observed Secondary wall deposition was associated with these MT patterns
G–J) Immunofluorescence microscopy of cells between the patches of epidermal cells at the perifery and the tracheary centres inside the protrusions MTs are oriented transverse to the axis formed by the epidermal patches and the tracheary centres
B basal side, C central side, E epidermal side, (Ep) facing epidermal patches, P protrusion, Pb preprophase band S spindle, arrow MT, arrow head ellipsiod pattern of MTs, open arrowhead lignin deposition, double arrow axis between epidermal patches and tracheary centre inside the protrusion Bar 10 μm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development (Hicks and Sussex 1970)

DISCUSSION

In tobacco explants protrusion development starts after 2 days of culturing in distinct regions at the basal side of the explants Cells in these regions mostly have randomly oriented MTs (Wilms & Derksen 1988)

Our results show that between 4–5 days after explantion patches of epidermal cells at the periery of the protrusion start to divide simultaneously with the formation of tracheary centres inside the protrusion After 6–7 days of culturing connecting tracheary elements are formed between the epidermal patches and the tracheary centre (see also Wilms & Sassen 1987) The orientation of MTs in the different parts of the protrusion reflects the direction of cell division In the shoot apex and the leaf primordium of *Vicia major* MTs tended to be arranged anticlinally in the tunica cells randomly in the corpus cells and transversely in cells of the rib meristem (Sakaguchi *et al* 1988) These findings are comparable to the arrangement of MTs in protrusions of tobacco explants At the patches on the epidermis MTs are oriented anticlinally and inside the protrusion MTs are oriented randomly At distinged regions of the protrusion cells are obeserved with transverse oriented MTs

One of the first visible signs of cell differentiation is organization of cortical MTs Orientation of MTs and place and plane of preprophase bands are marked vectors accompanying cell polarity Similar observations have been made previously not only in cortex cells of tobacco explants (Wilms & Derksen 1988) but also in epidermal cells of *Nautylocalyx lynchii* (Venverloo & Libbenga 1988) and in other cells (reviews Hepler & Palevitz 1974 Gunning & Hardham 1982)

MTs exist before the onset of spindle formation or of any other manifestation of mitosis Due to tensions created by directional growth of cells and tissues and to pressures exerted by the firm cell wall of plant cells spindles are directed parallel to and new cell walls are formed at right angles to the long cell axis (Stebbins 1986) In the patches of epidermal cells as in cells inside the protrusions the orientation of MTs reflects the direction of future cell division New polarity axes formed inside the protrusion are preceded by re-organization of MTs in these cells resulting in organized cell divisions

It is not yet known what is the earliest event in organogenesis but whenever this specific event has happened a cascade of reactions is started (for review see Stebbins 1986 Williams & Maheswaran 1986 Green 1988) One can only speculate on this first event However the result of this event must have its infuence on the organization of cortical MTs since MTs appear to be one of the first visible signs of cell commitment to cell polarity (Wilms & Derksen 1988)

It may be possible that factors like differences in membrane permeability create constant fluxes causing a positive pole of cations chiefly Ca^{2+} an H^{+} at one side and a negative pole at the opposite end of the cell or organ (Jaffe 1981 Nuccitelli 1983) As in embryogenesis a steady ionic current may be involved in determining and maintenance of cell polarity (Brawley et al 1984) Such currents may eventually lead to a re-grouping of hormone receptors in the cell membrane and subsequently differential gene activity and may also be involved in orientation of cortical MTs

In the biophysical model of Green (1988) changes in physical boundary conditions rather than any basic change in the cell rules may account for floral induction According to Green the basic cell rules are the result of a number of gene products encoded on one portion of the genome (termed A) identical for both vegetative and floral development Transition to flowering would then be activated by other parts of the genome (termed B and C) However in floral stalks of tobacco the tissue is already committed

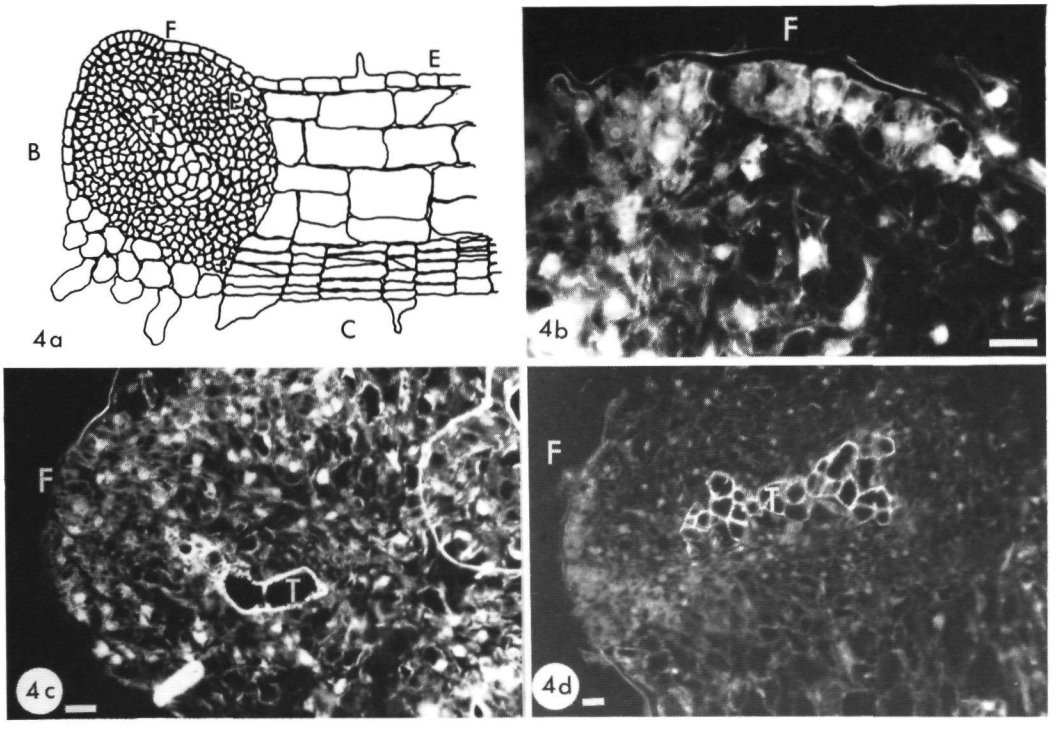

Fig.4. A) Drawing true to nature of light microscopic image of a longitudinal section of a 7 d old explant, showing the basal side of the explant with a protrusion area. Rectangles in drawing refer to Fig.3B–D.
B) Immunofluorescence microscopy of patches of epidermal cells.
C,D) Fluorescence microscopy of tracheary elements and patches of epidermal cells under ultraviolet light.
B: basal side; C: central side; E: epidermal side; F: floral primordium; P: protyrusion; T: tracheary centre. Bar: 20 μm.

to floral development, since floral buds can be initiated from flower stalks only. Previous results of Tran Thanh Van et al. (1985), showing initiation of both vegetative and generative buds have not been reproduced since.

In *Silene* and *Pisum*, the epidermis plays a crucial role in the initial stages of primordia formation (Lyndon & Cunninghame 1986). In tomato cotyledons, shoot differentiation starts at the perifery of callus (Monacelli et al. 1988). Possibly, in epidermal cells of tobacco explants, cell division is triggered followed by lateral inhibition, preventing neighbouring cells to react similarly (Green 1980; Williams & Maheswaran 1986). Changes in cation concentration can cause changes in organization of cortical MTs (Quader et al. 1986), resulting in the formation of patches of epidermal cells with cell division activity. As a result, more cell divisions may be induced in the sub-epidermal region of these patches, forming a meristem. Synthesis of e.g. auxin may take place in these meristematic

cells, inducing new polarity axes (Sachs 1984, Green 1988). Like in whole plants, local differences in auxin concentration and local auxin transport may induce bud formation.

In tobacco explants, auxin transport occurs from distal to proximal within 1 day (Smulders *et al.* 1988). Due to a different auxin concentration in the medium, floral buds are initiated at different sites of the explants. Cultured on low concentrations of auxin (10^{-7}N NAA), only at the basal side of the explants the auxin concentration is sufficient to initiate buds. On higher concentrations (10^{-6}M NAA), more regions of the explant have the ability to accumulate auxin and to initiate buds (Van Den Ende *et al.* 1984; Wilms & Sassen 1987; Smulders *et al.* 1988). Cultured on low concentrations of agar (less than 1% agar) more buds are formed, probably due to an increased availability of auxin to the explants (Chapter 3 of this thesis).

The first signs of tracheary element formation are orientation of cortical MTs in characteristic bands. No autofluorescence was observed in these cells, indicating that no secondary wall formation had occurred. After some time wall material (i.e. lignin) with autofluorescence activity is deposited in association with these bands. Similar results have been reported by Hardham & Gunning (1979) and Falconer & Seagull (1985). They showed that no deposition of Calcofluor white positive wall material occurred during the grouping of the MTs into bands. In the second stage Calcofluor white positive material was deposited in association with the MT bands.

Auxin is also involved in tracheary element formation (Sachs 1981). In tobacco explants auxin, originating from the meristem, initiates tracheary element formation from the tracheary centre to the perifery of the protrusion. Alternatively, local differences in cation concentration between the tracheary centre and the perifery of the protrusion may also induce the formation of new polarity axes (see also above). As tracheary centre formation and epidermal cell divisions occur at the same time, the tracheary centres inside the protrusion may result from exogeneous auxin in the medium, while the tracheary elements formed between the centre and the perifery of the protrusion may be a result of endogenous auxin or cation fluxes.

The situation described is very similar to that *in vivo*, where vascular bundles are located at a distance from the tip meristem, new tracheary elements are formed between buds and vascular bundles. Thus, tobacco explants are an excellent objects for future investigations on plant differentiation.

ACKNOWLEDGEMENTS.

I wish to thank Prof. Dr. M.M.A. Sassen, Dr. G.W.M. Barendse and Dr. J. Derksen for helpfull discussions and Dr. G.W.M. Barendse for correcting the English text.

REFERENCES.

Brawley SH, Wetherell DF, Robinson KR (1984) Electrical polarity in embryos of wild carrot precedes cotyledon differentiation. Proc. Natl. Acad. Sc. USA 81: 6064–6067.

Dustin P (1984) Microtubules. Springer, Berlin Heidelberg New York.

Falconer MM, Seagull RW (1985) Immunofluorescent and calcofluor white staining of developing tracheary elements in *Zinnia elegans* L. suspension cultures. Protoplasma 125; 190–198.

Green PB (1980) Organogenesis. A biophysical view. Annu. Rev. Plant Physiol. 31: 51–82.

----- (1988). A theory for inflorescence development and flower formation based on morphological and biophysical analysis in *Echeveria*. Planta 175: 153–169.

Goosen-de Roo L (1973) The relationship between cell organelles and cell wall thickenings in primary tracheary elements of the cucumber. I. Morphological aspects. Acta Bot. Neerl. 22: 279–300.

Gunning BES, Hardham AR (1982) Microtubules. Ann. Rev. Plant Physiol. 33: 651–698.

Hardham AR (1982) Regulation of polarity in tissues and organs. In: Lloyd, C.W. (ed). The Cytoskeleton in plant growth and develop-

ment pp 377–403 Academic press London
-----, Gunning BES (1979) Interpolation of microtubules into cortical arrays during cell elongation and differentiation in root of *Azolla pinnata* J Cell Sci 37 411–442
Hepler PK, Palevitz BA (1974) Microtubules and microfilaments Ann Rev Plant Physiol 25 309–362
Hicks GS Sussex IM (1970) Development *in vitro* of excised flower primordia of *Nicotiana tabacum* Can J Bot 48 133–139
Jaffe LF (1981) The role of ionic currents in establishing developmental pattern Phil Trans Roy Soc London B 295 553–566
Lyndon RF (1982) Changes in polarity of growth during leaf initiation in the pea *Pisum sativum* L Ann Bot 49 281–290
-----, Cunningham ME (1986) Control of shoot apical development via cell division Symposium Society for Experimental Biology 40 233–255
Monacelli B, Altamura MM Pasqua G, Biasini MG, Sala F (1988) The histogenesis of somaclones from Tomato (*Lycopersicon esculentum* Mill) cotyledons Protoplasma 142 156–163
Murashige T Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture Physiol Plant 15 373–397
Nuccitelli R (1983) Transcellular ion currents signals and effectors of cell polarity Modern Cell Biol 2 451–481
Quader H Deichgraber G Schnepf E (1986) The cytoskeleton of *Cobaea* seed hairs Patterning during cell-wall differentiation Planta 168 1–10
Roberts LW (1976) Cytodifferentiation in plants xylogenesis as a model system Cambridge University Press Cambridge
Sachs T (1981) Polarity changes and tissue organization in plants In Schweiger H G (ed) International Cell Biology 1980–1981 pp 489–496 Springer Verlag Berlin Heidelberg New York
----- (1984) Axiality and polarity In Positional controls in plant development, pp 193–224 Barlow P W & Carr, D J (eds) Cambridge University Press, London
Sakaguchi S, Hogetsu T, Hara N (1988) Arrangement of cortical microtubules in the shoot apex of *Vicia major* L Planta 175 403–411
Smulders MJM, Croes AF, Wullems GJ (1988b) Polar transport of 1-Naphthaleneacetic acid determines the distribution of flower buds on explants of tobacco Plant physiol 88 752–756
Stebbins GL (1986) Gene action and morphogenesis in plants In Gustafson, J P Stebbins, G L , Ayala, F J (eds) Genetics, development and evolution pp 29–46 17th Stadler Genetics Symposium Plenum Press, New York, London
Tran Thanh Van K (1977) Regulation of morphogenesis In Barz W, Reinhard E, Zenk MH (eds) Plant tissue culture and its bio-technological applications pp 367–385 Springer Berlin Heidelberg New York
Tran Thanh Van K Toubart P, Darvill AG Gollin DJ, Albersheim P (1985) Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins Nature 314 615–617
Van Den Ende G, Croes AF, Kemp A, Barendse GWM Kroh M, (1984) Floral morphogenesis in thin-layer tissue cultures of *Nicotiana tabacum* Physiol Plant 62 83–88
Venverloo CJ Libbenga KR (1987) Regulation of the plane of cell division in vacuolated cells I The function of nuclear positioning and phragmosome formation J Plant Physiol 131 267–284
Williams EG Maheswaran G (1986) Somatic embryogenesis Factors influencing coordinated behaviour of cells as an embryogenic group Ann Bot 57 443–462
Wilms FHA Derksen J (1988) Reorganization of cortical microtubules during cell differentiation in tobacco explants Protoplasma 146 127–132
----, Sassen MMA (1987) Origin and development of floral buds in tobacco explants New Phytol 105 57–65

IV. CELLULOSE MICROFIBRILS IN CORTEX CELLS OF TOBACCO EXPLANTS

CHAPTER 6.

ORIENTATION OF CELLULOSE MICROFIBRILS IN CORTEX CELLS OF TOBACCO EXPLANTS. EFFECTS OF MICROTUBULE-DEPOLYMERIZING DRUGS.

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SUMMARY

The orientation of nascent cellulose microfibrils (CMFs) at the cell wall of cortex cells was studied in relation to differentiation in explants of *Nicotiana tabacum*

In cortex cells of freshly cut explants the direction of CMFs is mostly transverse to the longitudinal axis of the cell After explantation, the synthesis of CMFs continues and they are deposited in new directions within 2 to 6 hours changing to parallel to the long axis of the cell Newly deposited CMFs are running in bundles After 1 to 2 days of culturing a thick layer of longitudinally oriented CMFs is formed in all cells These shifts occurred in not growing cells, since no visible changes in cell diameter or cell length could be observed

In cells of explants treated with ethylene, the changes in deposition of CMFs from transverse to longitudinal occurred within 2 hours after the onset of the experiment In these cells cortical microtubules (MTs) change their orientation from transverse to parallel to the cell axis also within 2 hours after the onset of the experiment In untreated cells, cortical MTs changed their orientation within 6 hours after explantation

In explants treated with colchicine or cremart, the orientation of CMFs changes within 1 hour and longitudinal microfibrils are found after 3 hours treatment In these cells, no cortical MTs were detected after one to three hours treatment

It is concluded that the orientation of CMFs in cortex cells of tobacco explants is not controlled by cortical MTs Both CMFs and MTs seem to be controlled by factors related to cell polarity

INTRODUCTION

Organogenesis can be studied experimentally *in vitro* using tissue explants (Tran Thanh Van 1977, Williams & Maheswaran 1986) The formation of new organs requires changes in cell polarity (reviews Green 1980 Hardham 1982) Marked factors accompanying the formation of new polarity axes in cells and tissues are changes in orientation of cortical microtubules (MTs) and cellulose microfibrils (CMFs) In elongating cylindrical cells, stomatal guard cells and during xylogenesis an especially high degree of congruence between MTs and CMFs is found (review Hepler 1985) This co-allignment does not necessarily mean that MTs control the orientation of CMFs Recent observations in algae and higher plants do not support this MT/CMF hypothesis (review Preston 1988)

Cultured under appropriate conditions, it is possible to induce flower development at predictable sites of tissue explants of *Nicotiana tabacum* (Van Den Ende *et al* 1984, Wilms & Sassen 1987) In a previous study we showed that, following explantation, marked changes occurred in the organization of the microtubular cytoskeleton These changes are thought to result from

de-differentiation of cortex cells Secondary changes occur only locally, and appear to predict the orientation of future cell divisions During the first hours of culturing cell elongation stopped and no changes in cell diameter were observed (Wilms & Derksen 1988)

In this study we examined the orientation of the newly deposited CMFs at the cell wall of cortex cells in tobacco explants before and after explantation, under several experimental conditions The results show positive correlation of the observed changes in CMF deposition with changes in cell polarity CMF orientation, however, appeared to be independant from changes in microtubular organization

MATERIALS AND METHODS

Preparation and Culture

Tissue strips, 0 6 by 7 mm were cut from floral stalks of *Nicotiana tabacum* and cultured on a Murashige and Skoog (M&S) medium (Murashige & Skoog 1962) supplied with 10^{-7}M NAA (1-naphthalene acetic acid) 10^{-6}M BAP (benzylamino-purine) 125 mM glucose and 1% agar On this medium floral buds will develop only at the proximal side of the explants Explants cultured on M&S medium supplied with 10^{-7}M NAA, 10 ^{7}M BAP 125 mM glucose and 1% agar did not develop floral buds and were used as a control (Wilms & Sassen 1987)

In order to examine a possible involvement of cortical microtubules on cellulose microfibril deposition several experimental conditions were used

To depolymerize MTs, explants were treated with

- 10^{-3}M, 10^{-4}M and 10^{-5}M Cremart (O-ethyl O-(3methyl-6-nitrophenyl) N-*sec*-butylphosphorothioamidate) for 1-3 hours, a substance with a structure and function similar to amiprophos-methyl (Mita & Shibaoka 1984)
- 10^{-1}M, 10^{-2}M and 10^{-3}M Colchicine for 1-3 hours
- cold treatment (4°C for 2 hours) appeared to be insufficient to depolymerize MTs

The direction of the microtubules was experimentally modified by fluxing explants with 10 ppm ethylene for 2 minutes

Fixation, Embedding and Sectioning

Explants were fixed in 4% Paraformaldehyde (PFA) in phosphatebuffer (pH 6 8) and 10% DMSO for 2 hours at room temperature After washing some explants were embedded in polyethylene Glycol 1500 (PEG) as reported earlier by Wilms and Derksen (1988) Other explants were quickly frozen in liquid Freon 22 and stored at -80°C

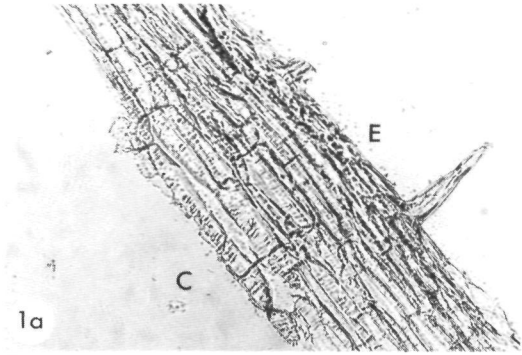
Longitudinal cryo-sections (15 μm thick) were cut with a cryo-cut microtome (Lameris cryo-cut microtome American Optical Corporation) and attached to clean glass slides coated with gela-

Fig 1 A B) Light micrograph of a longitudinal section of a freshly cut explant (A) and a 2 d old explant (B) The orientation of the pit-fields is transvese (A) and parallel (B) to the long cell axis Sometimes rounded pit-fields were found (B)

Fig 2 A) Electron micrograph of microfibrillar organization at the inner surface in cortex cell of a freshly cut explant CMFs are deposited transverse to the long cell axis
B) Relative frequency of CMFs on the inner surface in cortex cells of freshly cut explants The X-axis is parallel to the axis of the cell

Fig 3 A,C,D,E) Electron micrograph of microfibrillar organization at the inner surface in cortex cell of explant, 1 hour after explantation showing new directions of CMFs Smoothly curved CMFs (C E) and sharply curved CMFs (D,E) are observed
B) Relative frequency of CMFs on the inner surface in cortex cells of explants, 1 hour after explantation The X-axis is parallel to the axis of the cell

C central side of explant, E epidermal side of explant small arrow pit-field arrow cellulose microfibrils, open arrow long cell axis Fig 1 bar 5mm, Fig 2,3A bar 1 μm Fig 3C-E bar 0 1 μm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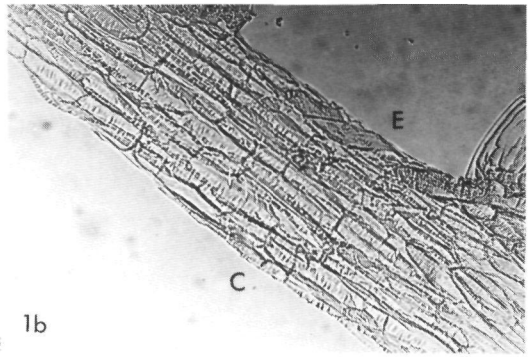
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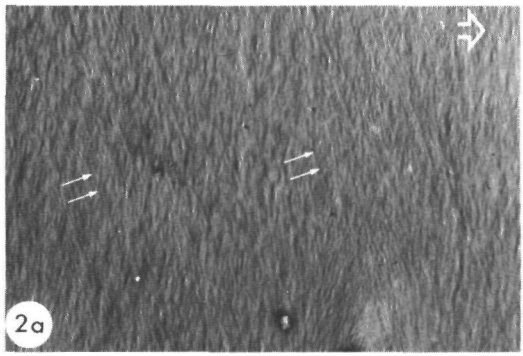
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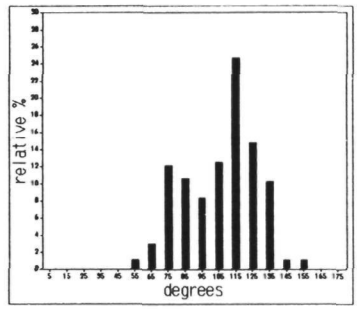
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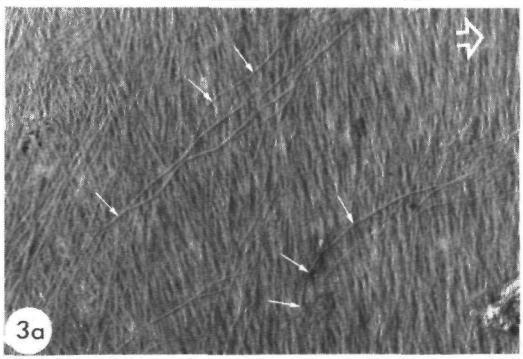
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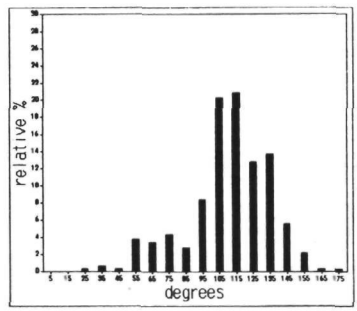
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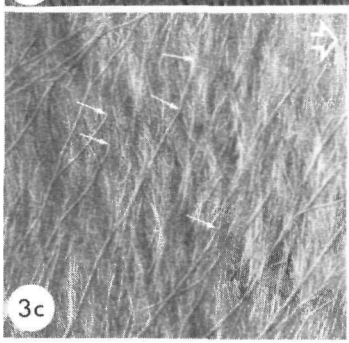
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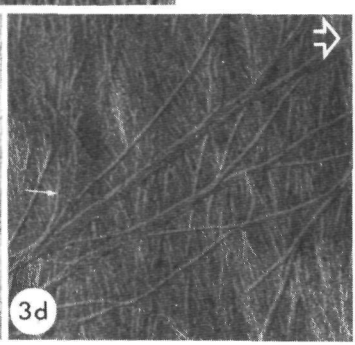
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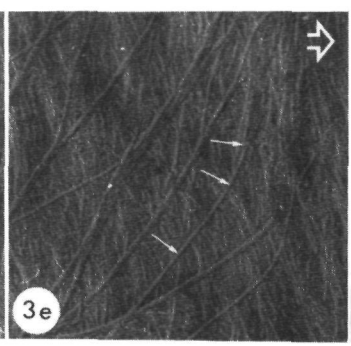
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tine

Longitudinal PEG-sections (15 μm thick) were cut with a steel knife on a Reichert hand microtome and attached to poly-L-lysine coated coverslips

Polarization Light Microscopy

Carefully washed cryo- or PEG-sections were extracted with hydrogen peroxide/glacial acetic acid (1/1,v/v) and birefringence of the cell walls was determined with a polarizing microscope (Leitz HM Pol) (see Ridge 1973 Preston 1974, Green 1980) The average direction of the cellulose in the wall was established from the extinction position in a polarizing microscope equiped with a Red I plate

Electron Microscopy

In order to visualize the cellulose microfibrils droplets of hydrogen peroxide/glacial acetic acid (1/1 v/v) were put over the cryo- or PEG-sections for several hours at 90°C After rinsing in water the sections were air-dried Replica s were made by shadowing the preparations with platinum at an angle of 45° and reinforced with carbon Parts with sections were selected and floated off in diluted HF and all biological material was removed with 40% $KMnO_4$ After rinsing in water the replicas were collected on formvar-carbon coated grids Examination was carried out with a JEOL CX 100 microscope

When cell strips were used instead of sections (Traas 1984 Sassen & Wolters-Arts 1986) similar results were obtained However since only few cells could be studied we preferred to use sectioned material

Quantitative Analysis

Micrographes of cell walls were printed at a final magnification of 60000 times Quantitative analysis was carried out by selecting CMFs with a trellis (distance of points in horizontal and vertical direction 1 cm) Next the angles of the CMF direction with reference to the axis of the cell were measured with a Kontron Videoplan computer as was reported previously by Sassen & Wolters-Arts (1986) From each time-period 10 cells were measured with about 100 CMFs per cell

Fluorescence Microscopy

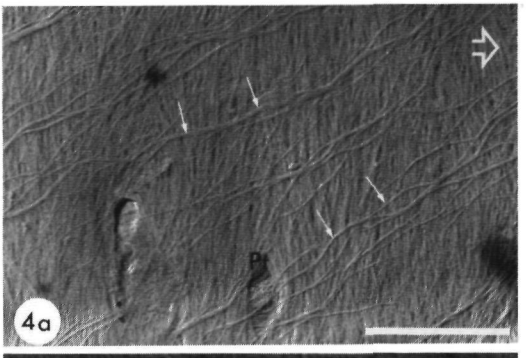
Cortical microtubules were detected on PEG-sectioned explants by using a monoclonal anti-tubuline (MAS 077b Sera Labs) as the first and a goat fluorescein isothyocianate (FITC)-labelled anti-rat immunoglobuline G (IgG) (Nordic Labs BV Tilburg the Netherlands) as a second antibody as previously reported by Wilms & Derksen (1988) The direction of cortical microtubules in cortex cells was determined using a protractor in the ocular of the microscope as previously reported by Wilms & Derksen (1988) Comparison between the orientation of MTs and CMFs were always made on sections from the same explants

Fig 4 A) Electron micrograph of microfibrillar organization at the inner surface in cortex cell of explant 2-4 hours after explantation CMFs are deposited in bundles Note the CMFs deposited over a pit-field
B) Relative frequency of CMFs on the inner surface in cortex cells of explants 2-4 hours after explantation The X-axis is parallel to the axis of the cell

Fig 5 A,C,D) Electron micrograph of microfibrillar organization at the inner surface in cortex cell of explant, 6 hours after explantation Longitudinal deposited CMFs are observed The underlying transverse layer of CMFs is still seen CMFs are deposited around and over pit-fields
B) Relative frequency of microfibrils on the inner surface in cortex cells of explants, 1 hour after explantation The X-axis is parallel to the axis of the cell

Fig 6 A,B) Electron micrograph of microfibrillar organization at the inner surface in cortex cell of explant 2 days after explantation A thick layer of longitudinally deposited CMFs is observed

Pt pit-field arrow cellulose microfibrils open arrow long cell axis Fig 4,5,6B bar 1 μm Fig 6A bar 0 1 μm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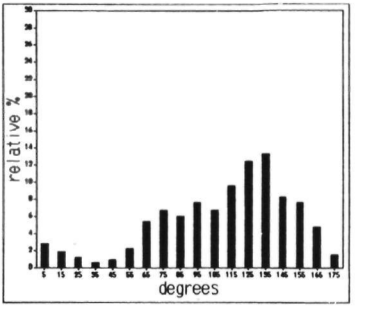
4a

4b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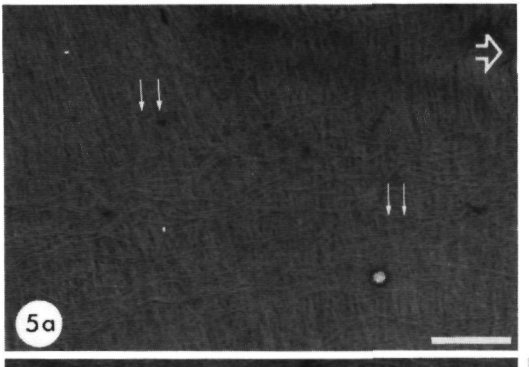
5a

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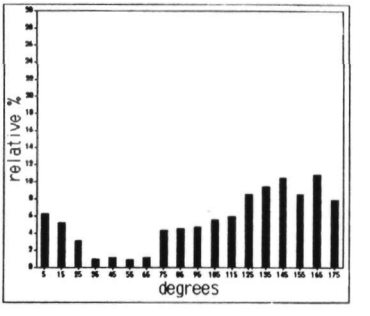
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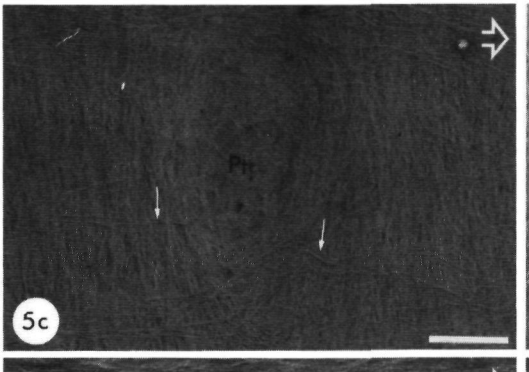
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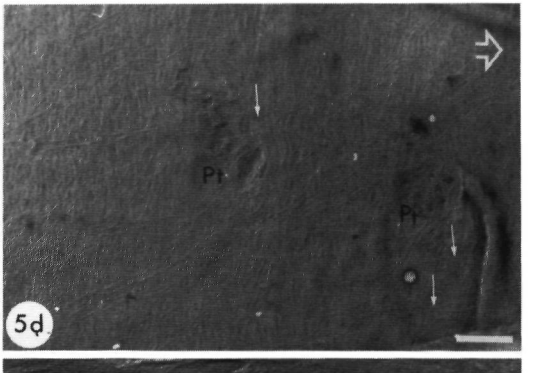
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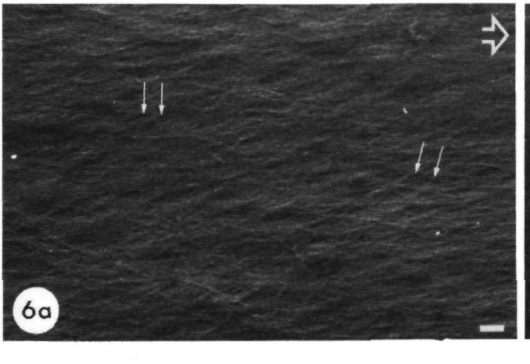
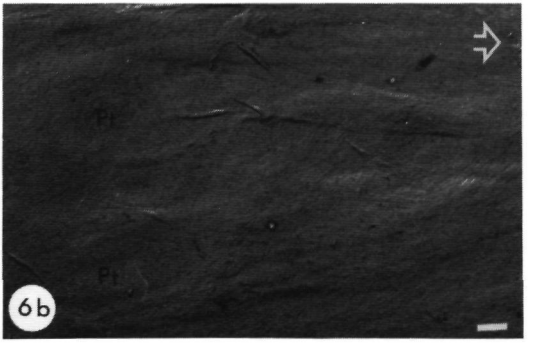

6a


Pt
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6b

RESULTS

Polarization Light Microscopy

In freshly cut explants the predominant orientation of the cellulose in the cell wall was transverse to the long axis of the cell Also the longitudinal axis of all pit-fields was transverse to the cell axis (Fig 1A) In 80% of the cells, the predominant orientation of the cellulose was still transverse to the long axis of the cell after two days of culturing, indicating that no visible changes had occurred In 20% of all cells, however, the predominant orientation was parallel to the longitudinal axis of the cell Some of these cells showed rounded pit-fields and others had their pit-fields with their longitudinal axis oriented parallel to the long axis of the cell (Fig 1B) Explants treated with ethylene cremart or Colchicine for 2 days showed no changes in the predominant orientation of the cellulose as compared to the orientation in untreated explants

Electron Microscopy

The extinction position in the polarization microscope does not represent the orientation of the last deposited cellulose microfibrils (CMFs), but gives the overall cellulose orientation Therefore we used tissue sections and replica techniques to visualize the newly deposited CMFs

In cortex cells of freshly cut explants the last deposited CMFs at the inner surface of the cell walls were running transverse to the longitudinal axis of the cell (Fig 2A) A thick layer of nearly parallelly aligned CMFs was seen They were composed of lamella, with alternated mean microfibril orientations of 75° to 85° (S-helix) and 95° to 105° (Z-helix) to the long axis of the cell (Fig 2B)

Within one hour after explantation individual CMFs were observed at the inner surface of the cell walls running in new directions (Fig 3A) Their mean directions were about 60° and 120° to the longitudinal cell axis (Fig 3B) Some CMFs were smoothly curved, whereas others showed more sharper angles (Fig 3C,D,E)

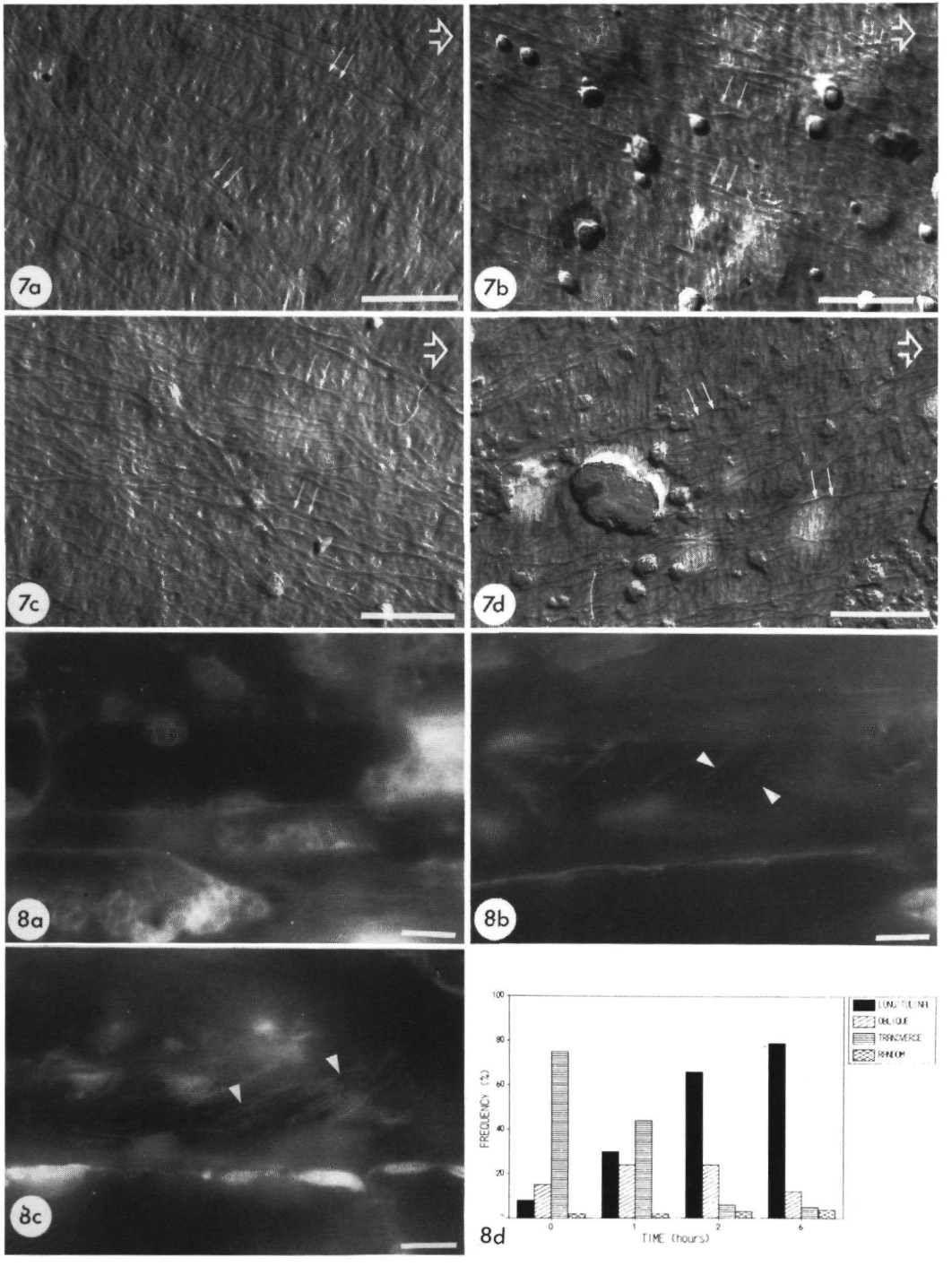
Explants cultured for 2 to 4 hours showed cortex cells with CMFs running in oblique and longitudinal directions (Fig 4A) The mean directions were about 45° respectively 135° to the long cell axis (Fig 4B) The underlying transverse layer of CMFs was still seen Also curved CMFs were visible Longitudinally oriented CMFs were always deposited over oblique or transverse ones CMFs were mainly running in bundles The distance between two adjacing bundles was about 0 5 μm The width of the bundles was about 0 25 μm consisting of 5-10 CMFs

After 6 hours the mean directions of CMFs

Fig 7 A) Electron micrograph of microfibrillar organization at the inner surface in cortex cells of explants treated with colchicine or with cremart for 1 hour CMFs are deposited in new directions
B,C) Same as Fig 7 A), only now treated with colchicine or cremart for 3 hours CMFs are deposited in new directions
D) Electron micrograph of microfibrillar organization at the inner surface of cortex cells of explants, treated with 10 ppm ethylene for 2 hours CMFs are deposited in new directions

Fig 8 A) Immunofluorescence microcopy of cortex cell of explant after treatment with high concentrations of colchicine or cremart for 1 hour No cortical MTs are visible
B) Immunofluorescence microcopy of cortex cell of explant after treatment with low concentrations of colchicine or cremart for 1 hour Cortical MTs are oriented oblique to parallel to the long cell axis
C) Immunofluorescence microcopy of cortex cell of explant after treatment with 10 ppm ethylene for 2 hours Cortical MTs are oriented oblique to parallel to the long cell axis
D) Orientation of cortical MTs in cortex cells of explants during a 1 to 6 hour treatment with 10 ppm Ethylene Number of cells 100/time SD 1-15%

arrow cellulose microfibrils, arrowhead microtubules, open arrow long cell axis Fig 7 bar 1 μm Fig 8 bar 5 μm


7a
7b
7c
7d
8a
8b
8c
8d
LONGITUDINAL
OBLIQUE
TRANSVERSE
RANDOM
FREQUENCY (%)
100
80
60
40
20
0
0
1
2
6
TIME (hours)

were 15° respectively 165° to the long cell axis (Fig 5A,B) CMFs were deposited not only around but also over pit fields (Fig 5C,D)

After 24 to 48 hours of culturing cells showed thick layers of CMFs running parallel to the long axis of the cell (Fig 6A B) No underlying transverse CMFs were visible The longitudinal axes of the pit fields were oriented parallel to the long axis of the cell (Fig 6B)

These changes occurred in explants grown both on bud inducing and on control medium Similar observations were found when explants were placed in water for several hours

Explants treated for 1 hour with high or low concentrations of cremart or with high or low concentrations of colchicine showed CMFs running transverse to oblique to the longitudinal cell axis (Fig 7A) After a 3 hour treatment with high concentrations of cremart or colchicine cells were found with CMFs running oblique to parallel to the longitudinal cell axis (Fig 7B C)

In ethylene treated explants a more rapid direction change of CMFs occurred compared with untreated explants Within 2 hours after explantation the mean angle of CMFs was 15° respectively 165° to the longitudinal axis of the cell (Fig 7D)

Fluorescence Microscopy of Cortical MTs

Freshly cut explants treated for one to three hours with 10^{-3}M or 10^{-4}M cremart, showed no cortical microtubules (MTs) in the cortex cells (Fig 8A) Cortical MTs were also absent in explants treated for one to three hours with 10^{-1}M or 10^{-2}M colchicine In cells treated with 10^{-3}M colchicine or 10^{-5}M cremart for 3 hours cortical MTs were observed running oblique to longitudinal to the long cells axis (Fig 8B)

In explants treated with ethylene, the orientation of cortical MTs changed within 2 to 4 hours after the onset of the experiment from transverse to longitudinal to the cell axis (Fig 8C D) The change of MTs direction in ethylene treated cells occurred some 2 to 4 hours earlier than in untreated explants (compare Fig 8D with Fig 2 in Wilms & Derksen 1988)

DISCUSSION

In cortex cells of freshly cut tobacco explants the orientation of the last deposited CMFs is transverse to the long cell axis CMFs are deposited in one fibril thick lamellae with mean angles of 80° and 100° to the longitudinal cell axis

Within 2 to 6 hours after explantation CMFs were deposited in new directions, the orientation changes from transverse to parallel to the long cell axis The CMFs are more or less smoothly curved and lie in bundles After 1 to 2 days a thick layer of CMFs was visible running longitudinal to the cell axis Similar shifts are observed in cortex cells of the style of *N tabacum* after cutting (Wilms onpublished results) Such shifts in CMF deposition have been shown previously (Lloyd 1982 1984) The fact that intermediate orientations of curved CMFs are found could mean that there is a continuous microfibril synthesis during cell differentiation It may be concluded that the lamellated texture results from continuous synthesis and deposition of CMFs in alternating lamellae In root cells of *Pisum sativum* CMFs are deposited also as lamellae with alternating CMF orientation In these cells however no intermediate directions of CMFs were found (Hogetsu 1986) This could mean that in these cells microfibrils are synthesized discontinuous during wall formation or that the curve CMFs were not visible

Bundle formation, as was found in cortex cells of tobacco explants was also observed in root cells of *P sativum* (Hogetsu 1986), in cotton fibres (Ryser 1985) and in *Zea mays* (Mueller & Brown 1982a) It implies a controlled deposition of CMFs Changes at distinct spots on the plasmamembrane rather than changes all over the plasmamembrane could be responsible for this forma-

tion (see also below)

After explantation, the elongation of the cells stopped Within 1–2 days the diameter of the cells increased (Wilms & Derksen 1988) Changes in the direction of CMFs, as well as shifts in the direction of cortical MTs precede a visible change in cell shape, since these changes in direction occur within 6 hours after explantation In root cells of *P sativum*, the orientation of CMFs changed from transverse to oblique to the long cells axis after cell elongation stopped (Hogetsu 1986) Recently Bergfeld et al (1988) reported a reorientation of CMFs in coleoptile segments of *Zea mays* after cessation of the supply of auxin, from transverse to longitudinal within 30 to 60 minutes Conversely CMFs changed their orientation back to transverse after auxin treatment In our experiments this polarity change is independent of auxin treatment It occurs in cells cultured on medium with and without auxin

Other factors may also influence polarity Ethylene changes the cell polarity (Lang *et al* 1982 Eisinger *et al* 1983) Treatment with ethylene showed rapid shifts in the orientation of CMFs and MTs (see also Ridge 1973 Lang *et al* 1982 Mueller & Brown 1982b, Roberts *et al* 1985) Therefore it is possible that wound-ethylene produced after cutting the explants and ethylene induced by auxin (review Imaseki 1985) may cause these polarity changes and reorientations It is not excluded that the internal auxin concentration changes after cutting the explants changing cell polarity In tobacco explants, ethylene treatment caused a decrease in sensitivity of the explants for auxin (Smulders 1989)

The orientation of CMFs and MTs in tobacco explants changes within a few hours after explantation From drug experiments it is concluded that CMFs are not controlled by cortical MTs Though MTs are absent, due to colchicine or cremart treatment, CMF synthesis and ordered deposition is continued Although CMFs and MTs change their direction from transverse to parallel to the longitudinal cell axis within the same time interval after explantation, they act independently When drugs are used, destroying the microtubular skeleton, CMF synthesis continues and the direction of CMFs changes from transverse to longitudinal to the long cell axis This is in contrast with earlier experiments with *Coleus* were colchicine treatment leads to disruption of the CMF pattern (Hepler & Fosket 1971) and with *Oocystis* were colchicine treatment did not cause new orientations of CMFs (Quader *et al* 1978, review Robinson & Quader 1982) In *Closterium*, a spatial relationship was found between MTs and CMFs in primary wall formation (Giddings & Staehelin 1988) Quader (1986) showed that in *Oocystis solitaria* the direction of CMFs is controlled by cortical MTs

Co-orientation of MTs and CMFs does not necessarily conclude that CMFs are controlled by MTs as was reported by Lloyd (1984) and Hepler (1985) Our findings are comparable with earlier findings in helicoidal cell walls of root hairs (Emons 1982) and recent findings in *Chaetomorpha moniligera* (Okuda & Mizuta 1987) and in *Boergesenia forbesci* and *Valonia ventricosa* (Hayano et al 1988) that MTs do not control the orientation of microfibrils In non-growing and non-dividing cortex cells however this is the first observation

The question arises what factors are responsible for the orientation of CMFs Vectors in the plasma membrane differences in the Ca^{2+} concentration (Quader et al 1986), number and density of particle rosettes (Emons 1985, Mizuta 1985, Ryser 1985), internal geometry (Roland et al 1987), electric fields (Preston 1988), turgor (Derksen 1986) may change both CMFs and MTs at the same time after changes in cell polarity It seems however, that MTs and CMFs act independently Both however may have a similar controlling factor during morphogenesis Changes in cell polarity are accompanied by changes in the orientation of MTs and CMFs

To solve the problems of regulation and control of CMFs and cortical MTs and cell polarity, it is important to examine the plasmamembrane more carefully This is one of the subjects under study in our laboratory

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REFERENCES.

Bergfield R, Speth V, Schopfer P (1988) Reorientation of microfibrils and microtubules at the outer epidermal wall of maize coleoptiles during auxin-mediated growth Botanica Acta 101 57-67

Derksen J (1986) Cytoskelet control of cellulose microfibril deposition In Vian B, Reiss D Goldberg R (Eds) Cell Walls 1986 Proceedings of the 4th Cell Wall meeting Paris 1986 Groupe Paris France

Eisinger W Croner LJ Taiz L (1983) Ethylene-induced lateral expansion in etiolated pea stems Kinetics cell wall synthesis and osmotic potential Plant Physiol 73 407-412

Emons AMC (1982) Microtubules do not control microfibril orientation in helicoidal cell wall Protoplasma 113 85-87

Giddings TH jr Steahelin LA (1988) Spatial relationship between microtubules and plasmamembrane rosettes during the deposition of primary wall microfibrils in *Closterium* sp Planta 173 22-30

Green PB (1980) Organogenesis A biophysical view Ann Rev Plant Physiol 31 51-82

Hardham AR (1982) Regulation of polarity in tissues and organs In Lloyd CW (ed) The Cytoskeleton in Plant Growth and Development pp 377-403 Academic Press, London

Hayano S, Itoh T, Brown RM jr (1988) Orientaion of microtubules during regeneration of cell wall in selected giant marine algae Plant Cell Physiol 29 785-793

Hepler PK (1985) The plant cytoskeleton In Robards AW (ed) Botanical Microscopy 1985 pp 233-262 Oxford Univ Press

-----, Fosket DE (1971) The role of microtubules in vessel member differentiation in *Coleus* Protoplasma 72 213-236

Hogetsu T (1986) Orientation of wall microfibril deposition in root cells of *Pisum sativum* L var Alaska Plant Cell Physiol 27 947-951

Imaseki H (1986) Ethylene In Takahashi N (ed) Chemistry of plant hormones pp 249-264 CRC Press, Inc Boca Raton Florida

Lang JM, Eisinger WR, Green PB (1982) Effects of ethylene on the orientation of microtubules and cellulose microfibrils of Pea epicotyl cells with polylamellate cell walls Protoplasma 110 5-14

Lloyd CW (ed) (1982) The cytoskeleton in plant growth and development Academic Press London

----- (1984) Towards a dynamic helical model for the influence of microtubules on wall patterns in plants Int Rev Cytol 86 1-35

Mita T Shibaoka H (1984) Gibberallin stabilizes microtubules in onion leaf sheath cells Protoplasma 119 100-109

Mizuta S (1985) Assembly of cellulose synthesizing complexes on the plasma membrane of *Boodlea coacta* Plant Cell Physiol 26 1443-1453

Mueller SC, Brown RM jr (1982a) The control of cellulose microfibril deposition in the cell wall of higher plants I Can directed membrane flow orient cellulose microfibrils? Indirect evidence from freeze-fractured plasma membranes of maize and pine seedlings Planta 154 489-500

----- ----- (1982b) The control of cellulose microfibril deposition in the cell wall of higher plants II Freeze-fracture microfibril patterns in maize seedling tissues following experimantal alteration with colchicine and ethylene Planta 154 501-515

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture Physiol Plant 15 373-397

Okuda K, Mizuta S (1987) Modification in cell shape unrelated to cellulose microfibril orientation in growing thallus cells of *cheatomorpha moniligera*, Plant Cell Physiol 28 461-473

Preston RD (1974) The Physical Biology of Plant Cell Walls Chapman and Hall Ltd London

----- (1988) Cellulose microfibril-orienting mechanisms in plant cell walls Planta 174 67-74

Quader H (1986) Cellulose microfibril orientation in *Oocystis solitaria* proof that microtubules control the alignment of terminal complexes J Cell Science 83 223-234

-----, Deichgraber G, Schnepf E (1986) The cytoskeleton in *Cobaea* seed hair development Patterning during cell-wall differentiation Planta 168 1-10

-----, Wagenbreth I, Robinson DG (1978) Structure synthesis and orientation of microfibrils V On the recovery of *Oocystis solitaria* from microtubule inhibitor treatment Cytobiologie 18 39-51

Robinson DG, Quader H (1982) The microtubule-microfibril syndrome In Lloyd CW (ed) The Cytoskeleton in Plant Growth and Development pp 109-126 Academic Press, London

Roland JC, Reis D, Vian B, Satiat-Jeunemaitre B, Mosiniak M (1979) Morphogenesis of plant cell walls at the supramolecular level Internal geometry and versatility of helicoidal expression Protoplasma 140 75-91

Ridge I (1973) The control of cell shape and rate of cell expansion by ethylene effects on microfibril orientation and cell wall extensibility in etiolated peas Acta Bot Neerl 22 144-158

Roberts IN Lloyd CW Roberts K (1985) Ethylene-induced microtubule reorientation mediation by helical arrays Planta 164 439-447

Ryser U (1985) Cell wall biosynthesis in differentiating cotton fibres Eur J Cell Biol 39 236-256

Sassen MMA, Wolters-Arts AMC (1986) Cell wall texture and cortical microtubules in growing staminal hairs of *Tradescantia virginiana* Acta Bot Neerl 35 351-360

Smulders (1989) Auxin regulation of flower bud formation in tobacco explants Role of concentration and sensitivity Thesis University of Nijmegen

Traas JA, Braat P, Derksen JW (1984) Changes in microtubule arrays during the differentiation of cortical root cells of *Raphanus sativus* J Cell Biol 34 229-238

Tran Thanh Van K (1977) Regulation of morphogenesis In Barz W, Reinhard E, Zenk MH (eds) Plant tissue culture and its bio-technological application pp 367-385 Springer, Berlin Heidelberg New York,

Van den Ende G, Croes AF, Kemp A, Barendse GWM, Kroh M (1984) Development of flower buds in thin-layer tissue cultures of *Nicotiana tabacum* Physiol Plant 62 83-88

Williams EG, Maheswaran G (1986) Somatic embryogenesis Factors influencing coordinated behaviour of cells as an embryogenic group Ann Bot 57 443-462

Wilms FHA Derksen J (1988) Reorganization of cortical microtubules during cell differentiation in tobacco explants Protoplasma 146 127-132

----- Sassen MMA (1987) Origin and development of floral buds in tobacco explants New Phytol 105 57-65

V. VISUALIZATION OF CYTOSKELETAL ELEMENTS AND CELLULOSE MICROFIBRILS IN CORTEX CELLS OF TOBACCO EXPLANTS

CHAPTER 7.

VISUALIZATION OF CYTOSKELETAL ELEMENTS AND CELLULOSE MICROFIBRILS IN CORTEX CELLS OF TOBACCO EXPLANTS.

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SUMMARY

A method to visualize membrane bound cytoskeletal elements and cellulose microfibrils in the same preparation in cortex cells of tobacco explants is described The method consists of sectioning Polyethylene glycol embedded material, critical point drying and cleaving

INTRODUCTION

It is possible with different techniques to visualize cytoplasmic elements like cytoskeleton and organelles, and cell wall elements like cellulose microfibrils (CMFs) separately Sassen and Wolters-Arts (1986) showed cortical microtubules (MTs) and CMFs in the same preparation in stamen hairs of *Tradescantia virginiana* after freeze substitution and sectioning Using ultrathin sections, the area of the cell to be examined is, however, very small With the introduction of the dry cleaving method for plant cells (Traas, 1984), it was possible to observe separately cytoskeletal elements (like MTs, actin filaments) coated pits, mitochondria endoplasmic reticulum and CMFs over a much larger cell area Since then different cells have been examined using this method various cell types of roots of different species (Traas et al 1984, Traas et al 1985, Emons & Traas 1986), pollen tubes (Derksen et al 1985), protoplasts of different species (Emons & Traas, 1986) and seed hairs (Quader et al 1986) Recently Traas and Derksen (1989) have shown MTs and CMFs in root hairs in the same preparation after dry cleaving

As reported by Traas (1984), this technique of dry cleaving is not suitable for cells with thick walls Also cells of tobacco explants were hard to cleave Therefore, we have attempted to develop an alternative method for visualizing cytoskeletal elements and cellulose microfibrils in these cells Wolosewick (1980) introduced the water soluble embedding medium Polyethylene glycol (PEG) for electron microscopy in animal tissue studies Later Hawes and co-workers (1983 1985) used the PEG technique for electron microscopic studies on plant material Also for immunocytochemistry, the PEG embedding technique was succesfully used on plant material (Van Lammeren *et al* 1985, Wilms & Derksen 1988)

We used a combination of PEG embedding and dry cleaving to examine the cytoplasmic elements (e g MTs, filaments, coated pits) and the cell wall in cortex cells of tobacco explants

MATERIALS AND METHODS

Preparation and Culturing

Tissue strips, 0 6 by 7 mm, were cut from flower stalks of *Nicotiana tabacum*, and cultured in Murashige and Skoog (M&S) medium (Murashige & Skoog 1962), supplied with 10^{-7}M NAA (1-naphthalene acetic acid), 10^{-6}M BAP (benzyl-amino-purine), 125 mM glucose and 1% agar as previously described by Wilms & Sassen (1987)

Fixation and embedding

Explants were fixed in 4% paraformaldehyde

and 0 25% glutaraldehyde in a microtubule (MT) stabilizing buffer with 0 1% Tannic acid and 0 01% Triton X 100 for 2 hours at room temperature and embedded in aqueous polyethylene glycol 1500 (PEG), as described by Wilms and Derksen (1988) The MT stabilizing buffer contained 50 mM phosphate buffer, 10 mM EGTA, 5 mM $MgSO_4$ and 10% DMSO Longitudinal sections, 5–15 μm thick, were cut with a steel knife on a Reichert hand microtome Sections were placed in water, to dissolve PEG and attached to poly-L-lysine coated nickel fold-over (oyster) grids

The grids were snapped together, post fixed in 0 5% OsO_4 for 1 hour, rinsed in water for 1 hour, stained with 0 5% uranyl acetate for 1 hour and after rinsing in water for 2 hours they were dehydrated slowly in ethanol After critical point drying, the twin-grids were opened and both halves were examined under a JEM CX 100 electron microscope

For visualization of cellulose microfibrils the grids were shadowed with platinum at an angle of 45^0 and reinforced with carbon

RESULTS AND DISCUSSION

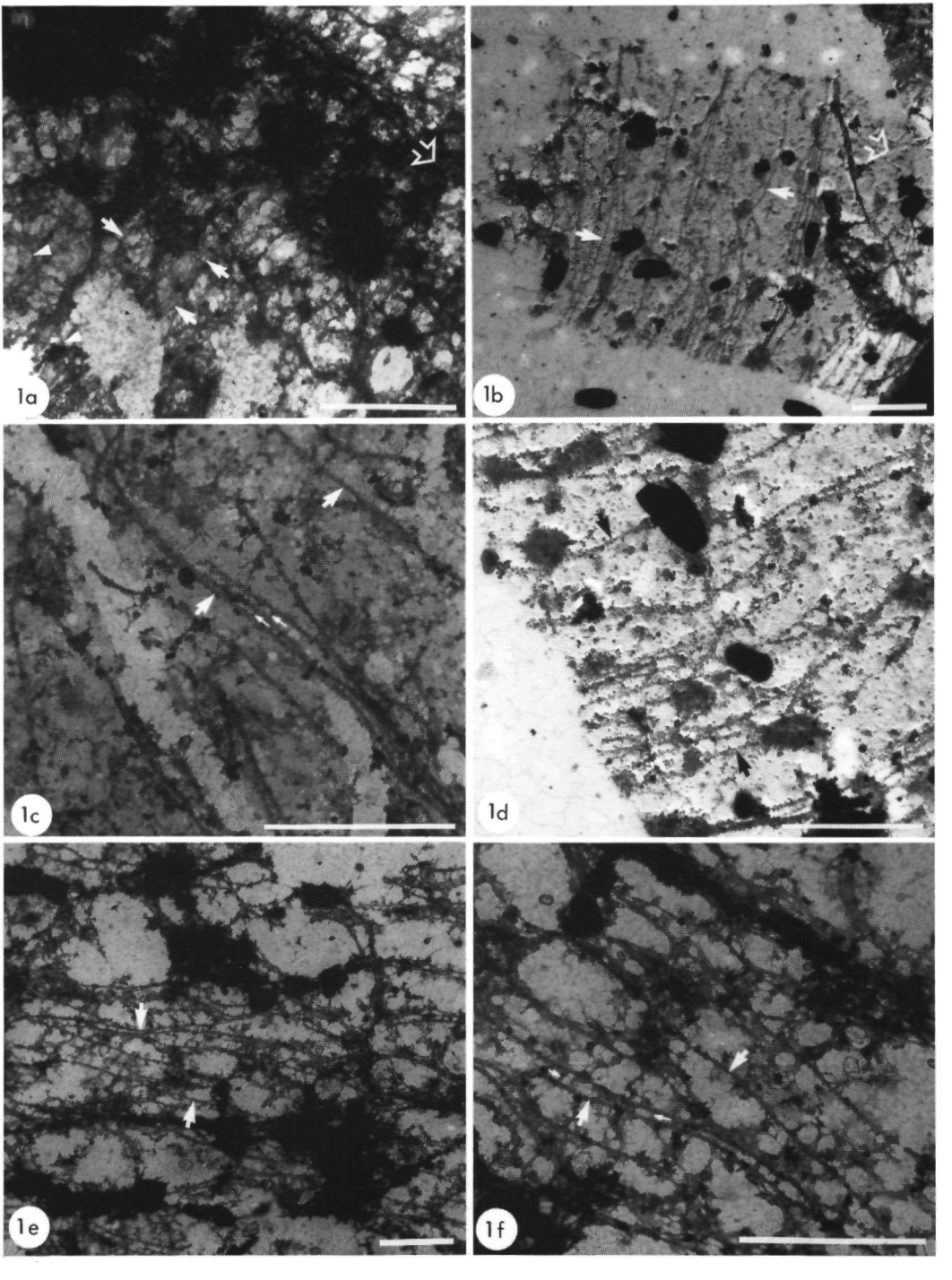
The overall view of the cells, embedded using the PEG method was essentially identical to that of cells after dry cleaving (Traas 1984) The ultrastructure of the cells was well preserved, as was reported also by Hawes & Horn (1985) for plant cells Large parts of the cells could be observed, with cytoskeletal elements, like MTs (Fig 1A–F,2D 2F,3A,3B) and smaller filaments (Fig 1A,2A–C), coated pits (Fig 2D–F) and organelles such as ER, mitochondria and many vesicles and with CMFs in the cell wall (Fig 3A–F)

With tannic acid added to the fixative, MTs were somewhat better preserved, as was reported also by Traas (1984) From 15 μm thick sections, better results were obtained and larger parts of the cells were visible than from 5 μm thick sections Sometimes cells, with large parts of the cytoplasm were seen Also smaller parts of the cell and sometimes only fragments of a cytoplasm was left on the grid

The difference with the method used by Hawes & Horn (1985) is that we used 15 μm thick sections instead of 0 25 – 1 μm sections and that we cleaved the sections by opening the oyster-grids instead of using only normal grids and not cleaving the preparations The advantage of our method is that both cytoplasm (Fig 1A–F,2A–F) and, at those places where cytoplasm is removed, CMFs in the cell wall can be examined (Fig 3A–F)

Whenever MTs and CMFs are observed simultaneously their orientation was identical In cells of freshly cut explants, the orientation of MTs and CMFs was transverse to the long cell axis After explantation cell polarity changed and the orientation of MTs and CMFs changed to parallel to the cell axis (see also Wilms & Derksen 1988 and chapter 6 of this thesis) During the first hours of culturing new and old lamellae of CMFs were visible Newly synthesized CMFs, running parallel to the long cell axis, were deposited over transverse CMFs (Fig 3A–B) The co-orientation of MTs and CMFs, however does not necessarely mean a control of the deposition of CMFs by cortical MTs, as was suggested by Lloyd

Fig 1 Electron micrograph from PEG embedded, sectioned, critical point dried and cleaved cortex cells of tobacco explants
A,C,E,F) Preparations before shadowing with Pt and reinforcement with C, showing microtubules (A,C,E,F) and filaments (A) and mitochondria (A)
B,D) Preparations after shadowing with Pt and reinforcement with C, showing microtubules
Arrow microtubule small arow connection between microtubules, arrowhead filament open arrow long cell axis, M mitochondria Bar 1μm


1a
1b
1c
1d
1e
1f

(1984) and Hepler (1985) Whenever MT depolymerizing drugs were used in tobacco explants, CMFs did change their orientation from transverse to parallel to the cell axis (see also chapter 6 of this thesis) It is concluded that in non-growing and non-dividing cells of tobacco explants the orientation of both MTs and CMFs are controlled by a yet unknown vector and that CMFs are not controlled by MTs

Thus PEG embedding and sectioning, followed by critical point drying and cleaving appears to be well suitable for the examination of cytoskeletal elements and CMFs in plant cells with thick cell walls Compared to the more conventional sectioning of resin and PEG embedded material for EM or HVEM (cf Hardham and Gunning 1979, Hawes and Horne 1985), this technique of sectioning and cleaving allowes not only to visualize substantially larger parts of the membrane surfaces, but also to study simultaneously the cytoplasm and CMF depositions in the cell wall

Microtubules

Cortical MTs appear as tubes running apart or in bundles of two or more tubes, sometimes connected to each other (Fig 1C,E,F) If no tannic acid was used during the fixation no tube structure was found In preparations before shadowing with platinum and reinforced with carbon, the diameter of MTs is 28 nm After Pt/C treatment, the diameter is about 35 nm (Fig 1B,1D,2F)

Probably due to the area of sectioning or cleaving, the length of the MTs was 0 5 to 3 μm, the mean length was 1 μm This is comparable to MTs in root tips of *Azolla* (Hardham & Gunning 1979) but is less than MTs in cortical cells of *Ceratopteris* (Traas 1984) The direction of the MTs was mostly perpendicular to the long cell axis as was previously reported by Wilms and Derksen (1988)

Filaments

In many preparations filaments with a diameter varying between 10–14 nm were found (Fig 2A,B,C) Their distribution was random and they appeared to form a three dimensional network Between these filaments, other filaments were observed with varying thickness, less than 9 nm (Fig 2A,B) The diameter of the filaments was measured in preparations before Pt/C treatment

Other Structures

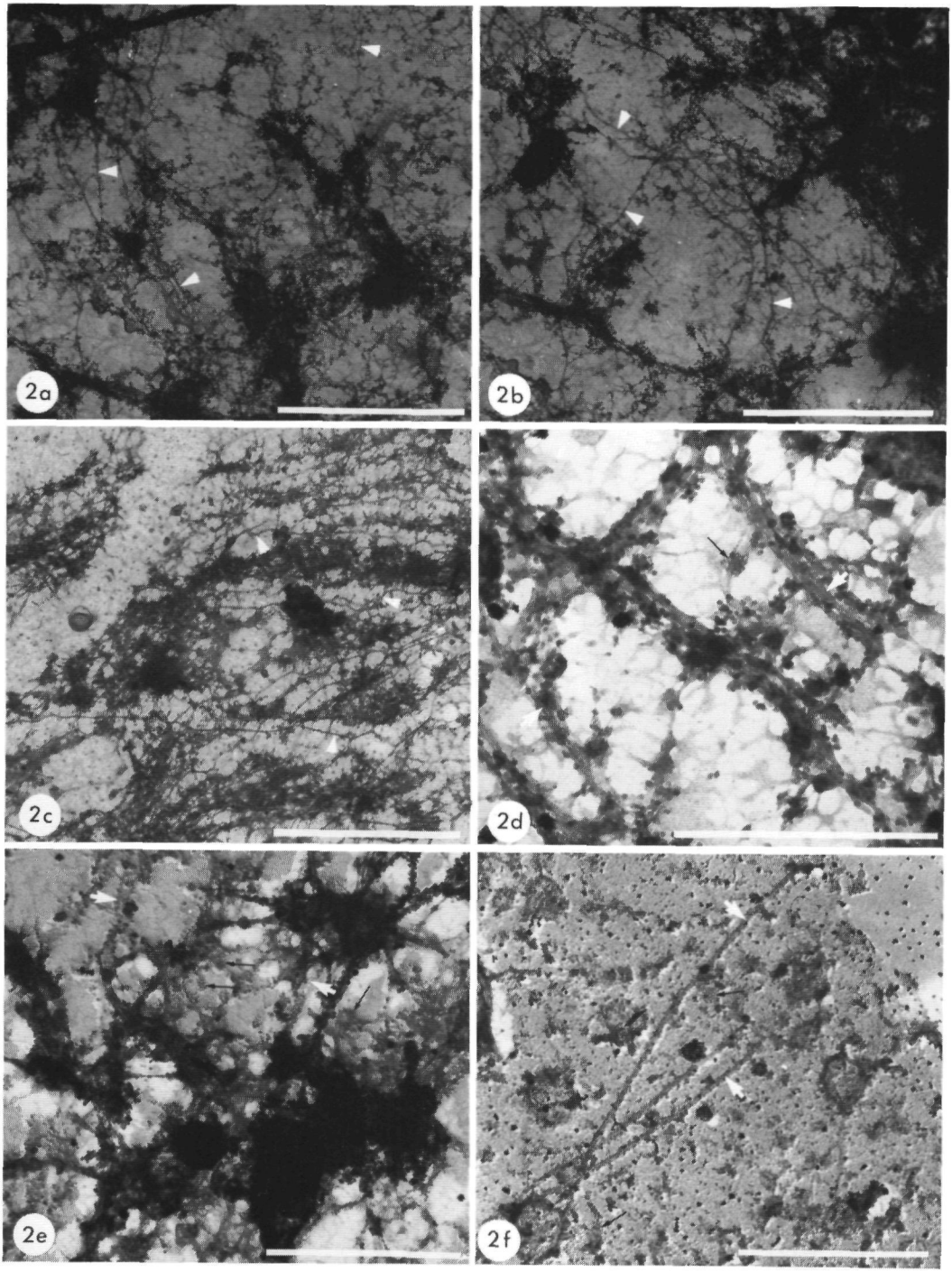
In some cells coated pits were frequently observed (Fig 2D,E,F) Sometimes it was difficult to identify coated pits on the plasmamembrane, partly because of the presence of structures on this surface that showed some similarity

Thus the present technique allowes visualization of coated pits and vesicles, though sometimes they may be blurred or covered by other cytoplasmic material

Cell Wall

At places were the cytoplasmic material was removed by cleaving or by sectioning, CMFs were visible It is clearly shown that the CMFs are deposited in lamellea with alternating CMF orientation forming a (poly-)lamellate texture (Fig 3C-F) Sometimes cytoplasmic material is left on the cell walls At the edges of the cytoplasm, remaining on the wall after cleaving, it is possible to observe simultaneously CMFs and MTs in the same orientation (Fig 3A B) The orientation of the

Fig 2 Electron micrographs from PEG embedded, sectioned, critical point dried and cleaved cortex cells of tobacco explants
A,B,C) Filaments of different size are visible in preparations without Pt/C treatment
D,E,F) Coated pits, vesicles and microtubules are visible in prepatations before (D) and after Pt/C treatment (E,F)
Arrow microtuble, arrowhead filament, small arrow coated pit Bar 1 μm


2a
2b
2c
2d
2e
2f

CMFs is mainly transverse to the longitudinal cell axis (Fig 3C–F) This is comparable to resent findings in extracted cell walls with hydrogen peroxide/gacial acetic acid (see chapter 6 of this thesis)

REFERENCES

Derksen J, Pierson ES, Traas JA (1985) Microtubules in vegetative and generative cells of pollen tubes Eur J Cell Biol 38 142–148

Emons AMC, Traas JA (1986) Coated pits and coated vesicles on the plasma membrane of plant cells Eur J Cell Biol 41 57–64

Hardham AR, Gunning BES (1979) Interpolation of microtubules into cortical arrays during cell elongation and differentiation in root of *Azolla pinnata* J Cell Sci 37, 411–442

Hawes CR Juniper BE Horne JC (1983) Electron microscopy of resin-free sections of plant cells Protoplasma 115 88–93

-----, Horne JC (1985) Polyethylene glycol embedding of plant tissues for transmission electron microscopy J Microsc 137 35–45

Helper PK (1985) The plant cytoskeleton In Robarts AW (ed) Botanical Microscopy 1985 pp 233–262 Oxford Univ Press

Lloyd CW (1984) Towards a dynamic helical modelfor the influence of microtubules on wall patterns in plants Int Rev Cytol 86 1–35

Murashige T Skoog F (1962) A revised medium for rapid growth and bioassys with tobacco tissue culture Physiol Plant 15 373–397

Quader H Deichgraber G Schnepf E (1986) The cytoskeleton of *Cobaea* seed hairs Patterning during cell-wall differentiation Planta 168 1–10

Sassen MMA, Wolters-Arts AMC (1986) Cell wall texture and cortical microtubules in growing staminal hairs of *Tradescantia virginiana* Acta Bot Neerl 35 351–360

Traas JA (1984) Visualization of the membrane bound cytoskeleton and coated pits by means of dry cleaving Protoplasma 119 212–218

-----, Braat P, Derksen J (1984) Changes in microtubule arrays during the differentiation of cortical root cells of *Rhaphanus sativus* Eur J Biol 34 229–238

-----, -----, Emons AM Meekes H, Derksen J (1985) Microtubules in root hairs J Cell Sci 76 303–320

Van Lammeren AAM, Keyzer CJ, Willemse MTM Kieft H (1985) Structure and function of the microtubular cytoskeleton during pollen development in *Gasteria verrucosa* (Mill) H Duval Planta 165 1–11

Wilms FHA Derksen J (1988) Reorganization of cortical microtubules during cell differentiation in tobacco explants Protoplasma 146 127–132

---- Sassen MMA (1987) Origin and development of floral buds in tobacco explants New Phytol 105 57–65

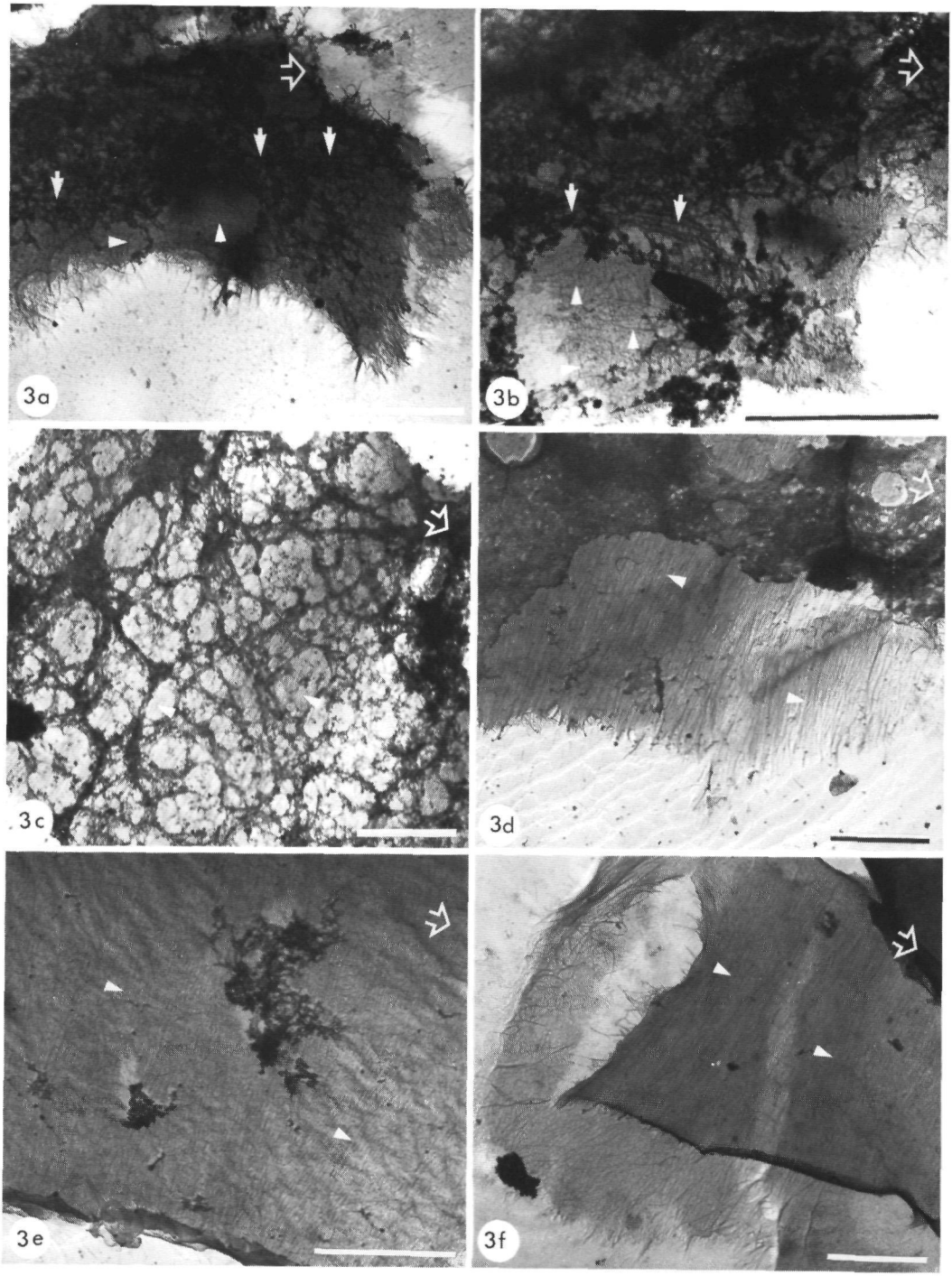
Wolosewick JJ (1980) The application of polyethylene glycol to electron microscopy J Cell Biol 86 675–681

Fig 3 Electron micrograph from PEG embedded, sectioned, critical point dried and cleaved cortex cells of tobacco explants Preparations are treated with Pt/C

A,B) Both microtubules and cellulose microfibrils are visible Microtubules and newly deposited microfibrils are orientated parallel to the long cell axis The underlying transverse oriented microfibrils are also visible

C,D,E,F) At places where cytoplasm is removed by cleaving cellulose microfibrils can be observed The direction of CMFs is transverse to the long cell axis

Arrow microtubules arrowhead cellulose microfibrils, open arrow long cell axis Bar 1 μm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VI. SUMMARY-SAMENVATTING

CHAPTER 8.

SUMMARY

Flower bud formation is an important event in the life of all plants Origin and development of floral buds is difficult to examine in intact plants, because of the variability of internal and external factores It is possible, however, to form floral buds on tissue strips, under controlable conditions Processes involved in floral bud formation can be studied easier

In this thesis morphogenesis and especially organogenesis of floral buds in tissue explants of *Nicotiana tabacum* is described Origin and development of floral buds is studied in relation to changes in cell polarity and establishment of new polarity axes Furthermore, the behaviour of cortical microtubules (MTs) and the deposition of cellulose microfibrils (CMFs) in the cells undergoing changes in cell polarity is discussed

Floral bud formation in tobacco explants, cultured on a Murashige and Skoog medium supplied with 10^{-7}M naphthalene acetic acid) 10^{-6} M benzyl-amino-purine, 125 mM glucose and 1% agar is a three-step proces The first stage (0-4 days) is characterized by protrusion formation (meristem-like regions) at the basal side of the explants resulting from epidermal and sub-epidermal cell divisions During the second stage (4-7 days), first signs of floral primordia are visible at the perifery of the protrusion and tracheary elements are developed inside the protrusion In the third stage new polarity axes are formed between the floral primordia and the tracheary elements Floral bud development is completed approximately 14 days after explantation (Chapter 2) In tobacco explants cultured on medium with 1% agar, floral initiation occurs within 4 days after explantation Within this time hormone concentrations inside the explants and sensitivity of the explants to hormones is optimal for bud formation After 4 days on a non-bud inducing medium, no floral buds can be initiated after transfer to a bud-inducing medium (Chapter 3)

Wound-healing is studied in cortex cells of tobacco explants adjacent to the medium Suberin is deposited after 4-7 days of culturing in these cells at the cut surface of the explants The number of cells containing suberin depends on the agar concentration of the medium In explants cultured on medium with a high concentration of agar (2%-5% agar) in all cells adjacent to the medium suberin is deposited Decreasing concentrations of agar results in a decreasing number of cells with suberin deposition On medium with 0 2% agar hardly any suberin containing cells are formed With decreasing agar concentration in the medium an increasing number of floral buds is formed on the explants Thus, in medium with low agar concentrations more hormone is available for the explants (Chapter 3)

The organization of cortical microtubules (MTs) in relation to cell polarity, establishm ent of new polarity axes and xylogenesis has been studied using indirect immunofluorescence on polyethylene glycol embedded tobacco explants Reorganization of cortical (MT), indicating de-differentiation of cells, occurs in all cells within 6 hours after explantation The directions of cortical MTs shifts from transverse to parallel to the longitudinal cell axis Cell polarity is changed from parallel to transverse to the long cell axis During the first 24 hours of culturing, no cell divisions and cell expansions occurs This change in MT direction is followed by further shifts that occur only locally and that predict the orientation of future cell divisions Re-differentia-

tion of cortex cells occurred after two days of culturing, and clusters of cells are formed at the proximal side of the explants, with randomly oriented cortical MTs These meristem-like regions represent the origin of the protrusions from which floral buds will develop Factors effecting the orientation of cortical MTs are discussed (Chapter 4)

Four to five days after explantation, cells at distinct patches at the perifery of the protrusions show MTs oriented transverse to the protrusion surface Anticlinal cell divisions occur, as the first signs of floral primordium formation A mersitem is formed underneath the epidermal patches of cell division Cells inside the protrusion show MTs oriented in circular or ellipsoid organizations Cell wall material is deposited associated with these MT bands and tracheary centres are formed Within six to seven days after explantation a new polarity axis is formed between the patches at the epidermis and the tracheary centre probably resulting from cation fluxes or local auxin transport between primordium and centre In this polarity axis, cells are observed with cortical MTs oriented transverse and cell divisions occur transverse to this axis The epidermis, the meristem underneath the epidermis and the tracheary centre inside the protrusion play an important role in the establishment of these axes (Chapter 5)

Chapter 6 describes the orientation of cellulose microfibrils (CMFs) at the inner surface of cortex cells in tobacco explants Six hours after explantation, the direction of CMFs is changed from transverse to parallel to the longitudinal axis of the cell After 1 to 2 days of culture a thick layer of longitudinally deposited CMFs is formed in all cells From experiments with MT-depolymerizing drugs (i e cremart and colchicine), it is concluded that the direction of microfibril deposition, in non-dividing and non-growing cortex cells of tobacco explants, was not controlled by cortical MTs Cell polarity is coupled to the organization of cortical MTs and CMFs Whenever cell polarity changes, it will effect the organization of both MTs and CMFs Factors affecting cell polarity and the deposition of CMFs are discussed

In chapter 7, a method for elctron microscopy is described to visualize membrane bound cytoskeletal elements and CMFs in the same preparation in cortex cells of tobacco explants The method consists of sectioning polyethylene glycol embedded tobacco explants, critical point drying and cleaving MTs filaments with different diameters coated pits and coated vescicles and CMFs are observed A co-orientation of cortical MTs and CMFs is found Changes in cell polarity will effect the organization of both cortical MTs and CMFs However, the direction of CMF deposition is not controlled by cortical MTs (see also chapter 6)

SAMENVATTING.

Bloemknopvorming is een belangrijk proces in de levenscyclus van een plant De bestudering van het ontstaan en de ontwikkeling van bloemknoppen aan de intacte plant wordt bemoeilijkt door de aanwezigheid van vele variabele interne en externe factoren Het is echter mogelijk om bloemknoppen te ontwikkelen op geisoleerde stukjes weefsel (weefsel explantaten), gekweekt onder controleerbare omstandigheden Processen die bij de bloemknopvorming betrokken zijn kunnen zo beter bestudeerd worden

In dit proefschrift worden enkele aspecten van morphogenese en in het bijzonder van organogenese van bloemknoppen, die ontstaan zijn op weefsel explantaten van *Nicotiana tabacum* (tabak), beschreven Het ontstaan en de ontwikkeling van bloemknoppen is onderzocht in relatie tot veranderingen van cel-polariteit en vorming van nieuwe polariteits-assen Eveneens is de organisatie van corticale microtubuli (MT) en de afzetting van cellulose microfibrillen (CMF) in de wand bekeken in relatie tot deze veranderingen van cel-polariteit

De vorming van bloemknoppen op explantaten van tabak, gekweekt op een Murashige en Skoog medium, met 10^{-7}M naphthaleen azijnzuur, 10^{-6}M benzyl-amino-purine, 125 mM glucose en 1% agar, kan verdeeld worden in 3 stadia Gedurende het eerste stadium (0-4 dagen) ontstaan er aan de basale zijde van het explantaat meristeem-achtige gebieden (protrusies), na epidermale en sub-epidermale celdelingen In het tweede stadium worden de eerste vormen van de bloemprimordia zichtbaar aan het oppervlak van de protrusies In het inwendige van de protrusies ontstaan houtvat elementen Het derde stadium wordt gekarakteriseerd door de vorming van polariteits-assen tussen de bloemprimordia en de houtvat elementen Ongeveer 14 dagen na explantatie zijn alle bloemdelen aangelegd (Hoofdstuk 2) Bloem-initiatie in explantaten, gekweekt op medium met 1% agar, vindt binnen 4 dagen na explantatie plaats Binnen deze tijd zijn de inwendige hormoon concentraties en de gevoeligheid van het explantaat voor hormonen optimaal voor bloemknopvorming Explantaten die 4 of meer dagen op een niet bloemknop-inducerend medium zijn gekweekt zullen, na overzetting op een bloemknop-inducerend medium, geen knoppen ontwikkelen (Hoofdstuk 3)

In hoofdstuk 3 wordt de wond-heling besproken van cortex cellen van tabaks-explantaten, die kontakt maken met het medium Binnen 4 tot 7 dagen wordt in deze cellen suberine afgezet Het aantal van de suberine bevattende cellen is afhankelijk van de agar concentratie in het medium Wanneer explantaten gekweekt worden op een medium met een hoge concentratie agar (2%-5% agar) is suberine afgezet in alle cellen, die kontakt maken met het medium Een verlaging van de agar concentratie in het medium heeft een verlaging van het aantal suberine bevattende cellen tot gevolg Explantaten gekweekt op medium met 0 2% agar vormen geen suberine bevattende cellen Een verlaging van de agar concentratie heeft ook tot gevolg dat het aantal bloemknoppen, die op een explantaat gevormd worden, toeneemt De beschikbaarheid van hormonen in medium met een lage agar concentratie is dus groter

Met behulp van indirecte immunofluorescentie op polyethyleen glycol ingebedde explantaten van tabak, is de organisatie van corticale MT bestudeerd in relatie tot cel-polariteit, ontstaan van nieuwe polariteits-assen en xylogenese

Binnen 6 uur na explantatie vindt in alle cellen van het explantaat re-organisatie van MT plaats Deze de-differentiatie wordt gekenmerkt door een verandering van organisatierichting van MT van dwars op de lengteas naar parallel aan de lengteas van de cel De cel-polariteit verandert van parallel aan de lengteas naar dwars op de lengteas van de cel Gedurende de eerste 24 uur van kweek treden er geen cel-delingen en geen celstrekkingen op Deze eerste richtingsveranderingen van MT worden gevolgd door nieuwe, lokaal optredende veranderingen, die de oriëntatie van de nieuw te vormen celwand aangeven Re-differentiatie van cortex cellen gebeurt na 2 dagen van kweek, waarbij clusters van cellen gevormd worden, aan de basale zijde van het explantaat In deze meristeem-achtige gebieden is de orientatie van corticale MT voornamelijk random Deze gebieden vormen de protrusies waarop de bloemknoppen zich zullen ontwikkelen De organisatie van corticale MT is een weergave van de cel-polariteit Factoren die deze orientatie van MT beinvloeden worden besproken (Hoofdstuk 4)

Vier tot vijf dagen na explantatie vertonen cellen op bepaalde plaatsen aan de periferie van de protrusie dwars georienteerde MT ten opzichte van het protrusie oppervlak Op deze plaatsen zullen anticlinale delingen plaatsvinden, als eerste tekenen van bloemprimordium ontwikkeling Cellen binnen de protrusie vertonen MT, die in cirkels of in ellipsen georienteerd zijn Celwand materiaal wordt afgezet geassocieerd met deze patronen van MT en een centrum van houtvat elementen wordt gevormd Binnen 6 tot 7 dagen na explantatie wordt een nieuwe polariteits-as gevormd tussen het epidermale gebied met celdelings-activiteit en het centrum van houtvat elementen Dit wordt waarschijnlijk veroorzaakt door cation fluxen of lokaal auxine transport tussen de epidermis en het centrum van houtvat elementen De organisatie van corticale MT in deze nieuwe polariteits-as staat dwars op deze polariteits-as De epidermis, het daaronder gelegen meristeem en het centrum van houtvat elementen spelen een belangrijke rol in de totstandkoming van deze polariteits-assen (Hoofdstuk 5)

In hoofdstuk 6 wordt de afzetting van CMF in cortex cells van weefselexplantatan van tabak besproken Binnen 6 uur na explantatie verandert de richting van afzetting van CMF van dwars op de lengteas naar parallel aan de lengteas van de cel Na 1 tot 2 dagen kweek is er een dikke laag van CMF gevormd, die parallel aan de cel-as georienteerd zijn Uit experimenten met MT-afbrekende stoffen (cremart en colchicine) kan geconcludeerd worden dat, in niet delende en in niet strekkende cortex cellen van tabak, de richting waarin CMF worden afgezet niet onder controle staat van corticale MT Cel-polariteit is gekoppeld aan de organisatie van MT en CMF Verandering van cel-polariteit heeft gevolgen voor de organisatie van zowel MT als ook CMF Factoren die invloed hebben op cel-polariteit en afzetting van CMF worden in dit hoofdstuk besproken

In hoofstuk 7 wordt een nieuwe methode behandeld waarin, onder de electronen microscoop tegelijkertijd membraan gebonden cytoskelet elementen en CMF zichtbaar gemaakt worden in cortex cellen van tabaksexplantaten De methode bestaat uit het snijden van coupes van polyethyleen glycol ingebedde explantaten, kritisch punt drogen en klieven MT, filamenten van verschillende diameter, "coated pits" en "coated vesicles" en CMF zijn zichtbaar Een co-orientatie van MT en CMF wordt gevonden De afzetting van CMF staat echter niet onder controle van corticale MT (zie hoofdstuk 6)

LIST OF PUBLICATIONS

Flik G, Aben W, Wilms F, Wendelaar Bonga SE K^+ and H^+–dependent Na^+–ATPase activity and active Na^+–transport in de gills of the fish *Sarotherodon mossambicus* In The third congress of the European Society for Comparative Physiology and Biochemistry 1981, Noordwijkerhout (Abstract)

Wilms F, Balm P, van der Mey JCA, Wendelaar Bonga SE Effect of exposure to acid water on osmoregulation and ultrastructure of skin and gills in the teleost *Oreochromis mossambicus* In The first congress of Comparative Physiology and Biochemistry 1984, Luik (Abstract)

Wilms FHA (1986) Origin and development of floral buds in tobacco explants Acta Botanica Neerlandica, 35 524 (Abstract)

Wilms FHA, Sassen MMA (1987) Origin and development of floral buds in tobacco explants The New Phytologist 105 57–65

Wilms FHA (1988) Reorganization of cortical microtubules in tobacco explants Acta Botanica Neerlandica, 37, 321 (Abstract)

Wilms FHA, Derksen J (1988) Reorganization of cortical microtubules during cell differentiation in tobacco explants Protoplasma, 146, 127–132

Wilms FHA (1988) Reorganization of cortical microtubules and cellulose microfibrils in cortex cells of tobacco explants European Journal of Cell Biology 47, 57 (suppl 24)

Lent PLEM van, Wilms FHA, van den Berg WB Interaction of polymorphonuclear leucocytes with articular cartilage of immobilized arthritic joints A scanning electron microscopic study Annals of Rheumatic Diseases (accepted for publication)

Wilms FHA, Neelissen J, Kroh M Suberization and bud formation in tobacco explants New Phytology (submitted) (Hoofdstuk 3 van dit proefschrift)

Wilms FHA Organization of cortical microtubules during floral bud formation Planta (submitted) (Hoofdstuk 5 van dit proefschrift)

Wilms FHA Wolters–Arts AMC Changes in orientation of cellulose microfibril deposition during differentiation of cortical cells in tobacco explants Protoplasma (submitted) (Hoofdstuk 6 van dit proefschrift)

CURRICULUM VITAE.

Frans Herman Antoon Wilms werd geboren op 10 juni 1957 te Tegelen In 1976 behaalde hij het diploma Atheneum-B aan het St Thomascollege te Venlo

In datzelfde jaar startte hij zijn studie Biologie aan de Katholieke Universiteit te Nijmegen Na het behalen van het kandidaatsexamen (B1g) in 1979 begon hij aan zijn eerste hoofdvak op de afdeling Dierfysiologie (tegenwoordig vakgroep Experimentele Dierkunde) onder leiding van Prof Dr S E Wendelaar Bonga Het betrof een fysiologisch onderzoek naar de invloeden van verscheidene externe en interne factoren op de water- en ionenhuishouding bij de teleost *Sarotherodon mossambicus* Van 1980-1981 was hij werkzaam op de afdeling Geobotanie (tegenwoordig Experimentele Plantenoecologie) onder leiding van Prof Dr V Westhoff, waar hij zijn tweede hoofdvak deed Tijdens dit hoofdvak heeft hij op de Boschplaat van Terschelling onderzoek verricht naar de relatie tussen vegetatiepatroon en milieudynamiek In 1982 heeft hij een bijvakstage gevolgd op de afdeling Hematologie, van het St Radboudziekenhuis te Nijmegen onder leiding van Prof Dr C Haanen Op deze afdeling verrichtte hij celbiologisch en biochemisch onderzoek aan het Common Acute Lymphoblastic Leukemia Antigen (CALLA) met behulp van CALLA-mRNA micro-injecties in oocyten van *Xenopus laevis* In 1983 behaalde hij het doktoraalexamen Biologie met onderwijsbevoegdheid

Na een jaar vrijwilligerswerk op de afdeling Dierfysiologie aanvaardde hij in 1984 de functie van wetenschappelijk assistent bij de vakgroep Experimentele Plantkunde, afdeling Submicroscopische Morfologie onder leiding van Prof Dr M M A Sassen Het betrof een 0 8 aanstelling waarbij hij de vrije vrijdagen gebruikte om zich op andere gebieden te bekwamen Zo behaalde hij in 1987 het AMBI-module I1 diploma Gedurende zijn aanstelling heeft hij verscheidene cursussen gevolgd Algoritmiek (Algol 68) bij de afdeling Informatica van de KUN in 1984, Deskundige Stralingshygiene niveau 3 bij de afdeling Universitaire Stralingsbeschermingsdienst van de KUN in 1985, "Immunolabelling for Electron Microscopy' aan de Post Graduate Medical School te London in 1986

Hij is gehuwd met Marlie Becks en ze hebben een dochter Susan

STELLINGEN

behorende bij het proefschrift

ORIGIN AND DEVELOPMENT OF FLORAL BUDS IN TOBACCO EXPLANTS
ORGANIZATION OF MICROTUBULES AND MICROFIBRILS IN DIFFERENTIATING CELLS

I

Verandering van richting van corticale microtubuli komt niet tot stand door verandering van richting van het bestaande microtubuli-skelet, maar door opbouw van microcrotubuli in een andere richting

Roberts IN, Lloyd CW, Roberts K (1985) Ethylene-induced microtubule reorientation mediation by helical arrays Protoplasma 137 56-62

Hoofdstuk 4 van dit proefschrift

II

De richting waarin cellulose microfibrillen worden afgezet wordt niet bepaald door de richting van corticale microtubuli

Hoofdstuk 6 van dit proefschrift

III

Celpolariteit wordt weerspiegeld door de organisatie van corticale microtubuli en cellulose microfibrillen

Hoofdstuk 4,5 en 6 van dit proefschrift

IV

In weefselexplantaten van tabak is de vorming van bloem primordia afhankelijk van het ontstaan van nieuwe polariteits-assen

Hoofdstuk 2 en 5 van dit proefschrift

V

Bloemknopvorming in weefselexplantaten van tabak is afhankelijk van de opname van hormonen aan de centrale zijde van het explantaat

Hoofdstuk 3 van dit proefschrift

VI

PEG helpt

VII

Resultaten uit dier experimenteel onderzoek kunnen niet ge-extrapoleerd worden naar de humane situatie, maar het is wel nuttig dat deze resultaten op hun waarde beoordeeld worden voor therapeutische toepassingen

Lent PLEM van, Wilms FHA, Berg WB van den Interaction of polymorphonuclear leucocytes with articular cartilage of immobilized arthritic joints A scanning electron microscopic study Annals of Rheumatic Diseases (in press)

VIII

De oorsprong van Perestroika en Glasnost in Rusland kan in 1984 gelegen hebben

IX

Generaliseren is nodig Weten is beter

X

Aangezien, volgens Lorenz, vogels in de eerste levensfase belangrijke informatie ontvangen over hun zang, lijkt het aannemelijk dat babies niet door ooievaars worden gebracht maar door kraaien

XI

Vanaf 1 september 1989 is uit een proefschrift in mindere mate de veelzijdigheid van een promovendus op te maken

XII

Gisteren zag ik het mooier

XIII

Een onderzoeker is als een ridder die op zoek is naar avontuur

XIV

De dikte van een proefschrift is minder afhankelijk van kwaliteit dan van vormgeving

XV

Een van de gevolgen van het instellen van een visitatie-commissie kan zijn dat de beoordeling van vakgroepen niet meer uitsluitend berust op het aantal wetenschappelijke publicaties maar ook op de kwaliteit van het onderwijs dat verzorgd wordt

NIJMEGEN, 20 JUNI 1989 F H A WILMS